Microscopic and molecular assessment of chlorhexidine tolerance mechanisms in *Delftia acidovorans* biofilms

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ABSTRACT

One of the most concerning characteristics of microbial biofilms is that of increased resistance to antimicrobial agents such as the commonly used biocide chlorhexidine (CHX). This can have huge impact on clinical, household and environmental settings. This is particularly alarming when it involves opportunistic pathogenic environmental organisms such as *Delftia acidovorans* as routine mitigation practices may fail to be effective.

This thesis examines tolerance mechanisms of *D. acidovorans* biofilms exposed to CHX at inhibitory and sub-inhibitory concentrations. To achieve the study goals and objectives, a CHX-tolerant *D. acidovorans* strain (WT15), (Minimum Inhibitory Concentration; MIC-15 μg ml^{-1}) was compared to a CHX-sensitive strain (MT51, MIC-1 μg ml^{-1}) that was obtained by mutating the wild type strain using transposon mutagenesis. Specific morphological, structural and chemical compositional differences between the CHX-treated and untreated biofilms of wild type and mutant strains were documented using microscopic techniques including confocal laser scanning microscopy (CLSM), scanning transmission x-ray microscopy (STXM), transmission electron microscopy (TEM) and infrared (IR) spectroscopy. Molecular level changes between biofilms formed by these two strains due to CHX treatment were compared using whole-cell proteomic analysis (determined using differential in-gel electrophoresis, or DIGE) along with fatty acid methyl ester (FAME) analysis.

The gene disrupted by transposon insertion that led to increased susceptibility to CHX in the mutant strain was identified as *tolQ*. CLSM revealed differences in biofilm architecture and thickness between the biofilms formed by strains WT15 and MT51. STXM analyses showed that WT15 biofilms contained two morpho-chemical cell variants; whereas, only one type was detected in MT51 biofilms. STXM and IR spectral analyses revealed that CHX-susceptible MT51 cells accumulated the highest levels of CHX, an observation supported by TEM wherein

prominent changes in the cell envelope of CHX-susceptible MT51 cells were observed. DIGE analysis demonstrated that numerous changes in protein abundance occurred in biofilm cells following CHX exposure and that most of these proteins were associated with amino acid and lipid biosynthesis, protein translation, energy metabolism and stress-related functions.

Overall, these studies indicate the probable role of the cell membrane and TolQ protein in CHX tolerance in *D. acidovorans* biofilms, in association with various proteins that are differentially-expressed.

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This thesis is dedicated to my father.

Miss you a lot.

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CHX	Chlorhexidine
CLS	Canadian Light Source
CLSM	Confocal Laser Scanning Microscopy
DIGE	Differential In Gel Electrophoresis
EDTA	Ethylenediaminetetraacetic acid
IR	Infra red
MIC	Minimum Inhibitory Concentration
MT	Mutant
NARMS	National Antimicrobial Resistance Monitoring System
NEXAFS	Near-edge X-ray Absorption Fine Structure
PCR	Polymerase Chain Reaction
QAC	Quaternary Ammonium Compound
STXM	Scanning Transmission Microscopy
SR-FTIR	Synchrotron radiation-based Fourier transformed infrared spectroscopy
TEM	Transmission Electron Microscopy
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
WT	Wild Type

1. INTRODUCTION

The majority of bacteria in nature live attached to surfaces in structured multicellular communities referred to as biofilms (Costerton *et al.,* 1995). They represent the sessile form of bacterial life versus a planktonic mode of existence. Biofilms have been associated with many persistent infections of humans and animals (Costerton *et al.,* 1999) and are a safety concern in the food industry (Wong, 1998). The root cause of the problems in industrial and medical settings due to biofilms is their ability to tolerate biocides, antibiotics, sanitising agents and host immune responses (Hoiby *et al.,* 2010a). It has been demonstrated that some bacterial biofilms can be as much as 1,000 times more resistant than their planktonic counterparts (Nickel *et al.,* 1985; Tremblay *et al.,* 2014). The mechanisms underlying antimicrobial tolerance in biofilms are not completely understood and hence a deeper understanding of them will help to develop compounds that can interfere with antimicrobial tolerance development and thus make biofilms susceptible to conventional, regularly used antimicrobial compounds. Biofilm resistance to biocides could be an intrinsic trait of the microorganism or could be acquired, or both (Russell, 1995). Intrinsic resistance could be a result of the nature and composition of outer cell layers, spore formation, and phenotypic adaptation, including biofilm formation and the production of degradative enzymes (Morente *et al.,* 2013). Genetic changes in a bacterial cell, either through mutation or by acquisition of extra-chromosomal elements such as plasmids or transposons, are molecular mechanisms of acquired resistance.

Among the commonly available biocides, chlorhexidine (CHX) is a widely-used broad spectrum antimicrobial agent and an active ingredient in many disinfectants, antiseptics and pharmaceutical preservatives. Being a cationic bisbiguanide, it exerts its effect by reacting with the bacterial cell membrane causing leakage or coagulation of cytoplasmic constituents (Hope and Wilson, 2004). Several microorgansims have been found to be resistant to CHX (Stickler and Thomas, 1980; Stickler, 2002); however, the exact mechanisms of CHX resistance are still not completely understood. Penetration failure within biofilms, the permeability characteristics of the bacterial outer membrane (OM), CHX degradation, and the presence of resistance genes or plasmids have all been reported as possible causes for CHX resistance (Gilbert and Moore, 2005; Tanaka *et al.,* 2005).

There are a number of food spoilage, pathogenic and environmental microorganisms that have been found to be associated with biofilms in food, medical, household and industrial environments. *Delftia acidovorans*, formerly known as *Comamonas acidovorans* and *Pseudomonas acidovorans*, is a Gram negative bacillus ubiquitously found in soil and water. Very little is known about the sessile form of *D. acidovorans* growth even though it has been occasionally associated with medical devices and water sources. *Delftia acidovorans* has been associated with several serious infections, including bacteremia, empyema, bacterial endocarditis, and ocular and urinary tract infections (Horowitz *et al.,* 1990; Cho and Lee, 2002; Perla and Kuntson, 2005) and thus may be emerging as an opportunistic pathogen. Studies on the effects of antimicrobial agents on *D. acidovorans* biofilms are very limited; however, resistance of *D. acidovorans* to some of the commonly used antibiotics has been reported (Chun *et al.,* 2009; Lipuma *et al.,* 2011; Kam *et al.,* 2012).

Qualitative and quantitative data can be derived from biofilms using various currently available microscopic techniques. These techniques have been very widely used to study the interactions of antimicrobial agents with microbial biofilms (Mangalappalli-Illathu and Korber, 2006; Dynes *et al.,* 2009; Tremblay *et al.,* 2014). Structure and formation of biofilms have been elucidated using various microscopic techniques like scanning and transmission electron microscopy. The application of confocal laser scanning microscopy (CLSM) has enabled the study of fully hydrated biofilms non-invasively and quantitatively. CLSM has the added advantage of being able to examine relatively thick biofilms by eliminating out-of-focus signals, allowing optical sectioning of biofilms and thereby the reconstruction of the 3D image of the entire biofilm structure (Lawrence *et al.,* 1991). Major biomolecules of biofilms can be mapped using soft X-ray scanning transmission microscopy (STXM) (Lawrence *et al.,* 2003). Speciation and distribution of metals and CHX have also been studied using this technique (Dynes *et al.,* 2006a; Lawrence *et al.,* 2012). Correlative microscopic studies, where two or more methods are employed on the same sample, have enabled researchers to get further insights into antimicrobial interactions with biofilms (Lawrence *et al.,* 2003; Dynes *et al.,* 2009).

Various proteomic and genomic studies have demonstrated the role of molecular mechanisms in bacterial

responses to antimicrobial agents. A number of genes are being added to the plethora of already reported genes that have been found responsible for biofilm formation and molecular adaptive mechanisms in various microbial species (Blair *et al.,* 2015). Molecular adaptive mechanisms may act either singly or synergistically to confer resistance, the most commonly reported include those: i) resulting in persisters cells, ii) involved in stress response, iii) starvation and dormancy, iv) detoxification, v) altered permeability of the outer membrane and vi) active efflux of the agent (Blair *et al.,* 2015).

This thesis consists of two major studies that examined the mechanisms of CHX tolerance in *Delftia acidovorans* biofilms, and are entitled:

(1) CHX tolerance in *D. acidovorans* biofilms - microscopic and spectroscopic analyses, and

(2) Proteomic analyses of CHX tolerance in *D. acidovorans* biofilms

The research work was based on the following hypothesis and techincal objectives

1.1 Hypotheses

There is a dearth of information related to mechanisms of CHX tolerance in bacterial species and particularly amongst the environmental species. In order to evaluate the possible mechanisms of CHX resistance in *D. acidovorans* biofilms, the following three hypotheses were considered for this research work. 1) CHX tolerance exists in environmental microorganisms such as *D. acidovorans,* and genetic status (or determinants) influences the susceptibility levels; 2) The cell membrane plays an important role in CHX tolerance in *D. acidovorans* biofilms and influences the uptake of CHX into the cell and structural/physiological effects thereon; 3) CHX tolerance in *D. acidovorans* biofilms is an interplay between several genes at the molecular level.

1.2 Technical objectives

1. To screen a CHX tolerant strain of environmental *D. acidovorans* and to develop a screening assay to isolate a CHX-sensitive mutant strain that was mutated using transposon mutagenesis.
2. To determine the gene or genes responsible for CHX tolerance.
3. To evaluate structural and chemcial changes in CHX tolerant and CHX-susceptible *D. acidovorans* biofilms when challenged with CHX using microscopic and spectroscopic analyses.
4. To examine the general stress response in *D. acidovorans* biofilms by comparing the proteomic state of CHX tolerant and CHX-susceptible strains when challenged with CHX.

1.3 Organization of thesis

This thesis is organized according to "manuscript-style" option of the College of Graduate Studies and Research.

2. REVIEW OF LITERATURE

2.1. Biofilms

The early twentieth century saw the emergence of studies focusing on living bacteria that grew on surfaces in the sessile state. Antonie van Leeuwenheok was the first to study surface-attached bacteria that were found on teeth plaque in 1684 (Rasmussen, 2000). The sessile growth of marine diatoms was first reported by Wilson (1925) and later in fresh water bacteria (Henrici, 1933). Many of the bacteria found in seawater would grow in a sessile state by attachment to a solid surface influencing their activity (Zobell, 1943). Microbes that grow on surfaces are commonly referred to as microbial biofilms. Microbial biofilms have been defined as populations of microorganisms that are attached to a solid substrate and typically surrounded by an extracellular polymeric substance (EPS) matrix (Costerton *et al.,* 1978). It is generally assumed that complex biofilm communities may have developed from planktonic cells as a result of unfavourable conditions that existed in primitive earth. There are several lines of evidence regarding biofilm formation in the fossil record as early as approximately three billion years ago (Rasmussen, 2000; Westall *et al.,* 2001). It is now well known that in a wide variety of natural habitats, the majority of microbes exist as biofilms (> 90% of microorganisms) and not as free-floating organisms (Costerton *et al.,* 1995; 1999).

Advances in research techniques, which will be discussed later in this chapter, have shown that biofilms are not simply a group of cells that are attached to surfaces, but are structurally- and dynamically-complex biological systems with substantial benefits to the community, for example, being 10 to 1000 times more resistant to stress than their free-existing counterparts (Mah and Toole, 2001; Hoiby *et al.,* 2010a). The EPS matrix in which the biofilm cells are aggregated consists of macromolecular components such as (primarily) polysaccharides, proteins, lipids, and extracellular DNA. This matrix plays an important role in the formation and maintenance of the biofilm structure and in defense mechanisms by protecting the cells from antimicrobial agents and host defenses in medical biofilms (Sutherland, 2001; Branda *et al.,* 2005; Ryder *et al.,* 2007). Apart from these two altered phenotypic differences (i.e., decreased susceptibility to antibiotics and predator/phagocyte tolerance)

between the bacterial aggregates and their planktonic counterparts, another density-dependent trait is bacterial cell-to-cell communication, known as quorum sensing (Fuqua *et al.,* 1994; Davies *et al.,* 1998). Quorum sensing (QS) is a system wherein bacteria sense signal molecules that are produced proportional to the density of cells located on the surface, and thereby initiates the expression of QS-regulated genes. Virulence and antibiotic tolerance have been found to be regulated by QS (Latifi *et al.,* 1995; Bjarnsholt *et al.,* 2005).

The occurrence of biofilms in hospital, marine, agricultural, industrial and food environments are a well-known phenomenon wherein multi-species biofilm communities predominate. Medical biofilms associated with acute infections have been extensively studied and reported (Costerton *et al.,* 1999; Donlan and Costerton, 2002). These acute infections caused by biofilm forming bacteria are more difficult to treat and so can develop into a more chronic disease state. Single species biofilms have been reported in hospital-related infections and medical devices and implants (Donlan and Costerton, 2002). *Pseudomonas aeruginosa* is the model organism used in many microbial biofilm-related studies. In human beings, biofilms formed by *P. aeruginosa* have been commonly associated with cystic fibrosis (Hoiby, 1974; 2002; Hoiby *et al.,* 2010b). Other infections associated with biofilm forming bacteria are periodontitis, chronic wounds, otitis media, endocarditis, and urinary tract infections. Biofilms may also cause persistent contamination of medical fluids in devices such as dialysis machines, venous catheters, dental water lines, contact lenses, airway ventilators, and indwelling devices such as prostheses and heart valves (Donlan and Costerton, 2002). Some other classic examples of environmental biofilms that are usually composed of multiple bacterial species include those found in drinking water lines, oil pipelines, wastewater treatment plants, the rhizophere, dental plaque, and human intestine and urinary systems (Donlan and Costerton, 2002; Parsek and Fuqua, 2004; Srey *et al.,* 2013). When cells are shed from biofilms into the overlying liquid, contamination of drinking water and subsequent entry into the food chain may result. Biofilms are a major source of food safety concerns in various food industries as they render microbes of concern resistant to routinely used cleaning chemicals (Srey *et al.,* 2013).

Biofilm formation is a very complex process and is an outcome of several physiological and genetic factors.

Davey and O'Toole (2000) have summarized the stages of biofilm development, and include: initiation, maturation, maintenance and dissolution. Hydrodynamic conditions of the surrounding flowing media, such as laminar and turbulent flow, have been shown to influence biofilm structures and alter these in response to changing flow conditions (Stoodley *et al.,* 1999; Davey and O'Toole, 2000). Biofilms grown under laminar flow conditions were found to be patchy, consisting of cell aggregates separated by interstitial voids; whereas, the unique characteristic of biofilms grown in turbulent flow was the presence of elongated "streamers" that oscillated in the bulk fluid. Nutrient availability (e.g., type of carbon source, osmolarity of the medium, etc,) and the type of abiotic surface (stainless steel, glass, PVC, etc.) can also influence biofilm formation (O'Toole and Kotler, 1998), creating variations from the "mushroom" model to flat, homogenous structures (Klausen *et al.,* 2003). Certain attachment surfaces can be rough or irregular (e.g., water pipes) while others can be smooth (e.g., catheters). Apart from the characteristics of the substratum, the nature of the bacterial cell surface can also play an important role in biofilm initiation and attachment. Cellular appendages such as flagella, pili, and fimbriae have been shown to facilitate microbial attachment to varying degrees (Rosenberg *et al.,* 1982; Korber *et al.,* 1989).

Several researchers have carried out a variety of studies on biofilms growing in aquatic habitats like freshwater, streams, rivers, etc. (Neu and Lawrence, 1997; Manz *et al.,* 1999; Battin *et al.,* 2003; Lawrence *et al.,* 2004; Rickard *et al.,* 2004; Lawrence *et al.,* 2005) and also by using laboratory media (Korber *et al.,* 1994; Anderl *et al.,* 2000; Purevdorj *et al.,* 2002). These include studies on the structure and pattern of biofilms formed, types of microorganisms involved, fluid dynamics, impact of nutrients, antibiotics, biocides and effects of other environmental factors such as pollutants. A variety of model systems may be used to cultivate microbial biofilm communities including chemostats, nutristats, gradostats, flow cells, rotating annular bioreactors or the microstat (Caldwell *et al.,* 1997).

2.2. *Delftia acidovorans*

Delftia acidovorans is a Gram negative, aerobic, non-fermentative, non-spore forming, motile bacillus, ubiquitously found in soil and water. It has also been isolated from animal sources, food, activated sludge, hospital equipment and human clinical specimens. It was named after the city of Delft, Netherlands, the site of the initial isolation of the type species (Wen *et al.,* 1999). It belongs to the family Comamonadaceae under the β subclass of the *Proteobacteria.* It has been known as both *Comamonas acidovorans* and *Pseudomonas acidovorans.* In 1999, it was renamed as *D. acidovorans* based on rRNA relatedness studies and is currently classified in the Pseudomonas rRNA homology group III (Wen *et al.,* 1999). The cells are straight to slightly curved rods (0.4-0.8 x 2.5-4.1 μm) and occur singly or in pairs (Wen *et al.,* 1999).

Generally, *D. acidovorans* has received little attention compared to bacteria such as other *Pseudomonas* species and *Escherichia coli*, and relatively little is known about its pathogenicity, prevalence, health and environmental risks, genetic information, antibiotic resistance profile and resistance mechanisms. The reason for the paucity of data is unknown but it may be suggested that the Comamonads were previously classified as Pseudomonads and were not studied or reported separately. The first detailed report of the association of this organism with a clinical infection and its identification as a pathogen appeared when it was isolated from a case of endocarditis in a 42-year old intravenous drug abuser (Horowitz *et al.,* 1990). Since then, reports of *D. acidovorans* association with a number of other serious infections, including bacteremia, empyema, bacterial endocarditis, ocular and urinary tract infections (del Mar Ojeda *et al.,* 1999; Cho and Lee, 2002; Perla and Kuntson, 2005) have been increasing, thus establishing this organism as an emerging opportunistic pathogen. *Delftia acidovorans* has also been implicated in many infections associated with hospital devices like vascular catheters (Ender *et al.,* 1996; Kawamura *et al.,* 2011), pressure-monitoring devices (Weinstein *et al.,* 1976) and surgical instruments (Mino *et al.,* 2000) as a consequence of surface growth. *Delftia acidovorans* has also been isolated from contaminated water sources (Stiegler *et al.,* 2003; La Duc *et al.,* 2004). Interestingly, *D. acidovorans* was also named as one of the bacterial agents transmitted from fish used as food, or by fish-handlers, thus establishing its presence in the food

industry as well (Novotny *et al.,* 2004).

Delftia acidovorans is often resistant to aminoglycosides, a class of drugs commonly used to treat Gram-negative bacterial infections, but usually susceptible to broad-spectrum cephalosporins, trimethoprim-sulfamethoxazole, fluoroquinolones, and tetracycline (Chun *et al.,* 2009; Lipuma *et al.,* 2011). A *D. acidovorans* strain that caused keratitis in a patient showed slow response to vancomycin and amikacin but was successfully treated with ceftazidime (Lee *et al.,* 2008). *Delftia acidovorans* isolated from a urinary tract infection was only susceptible to cefoperazone/sulbactam, imipenem, and doripenem but was resistant to all aminoglycosides, quinolones, as well as third- and fourth-generation β-lactam antibiotics (Kam *et al.,* 2012). In this report, they cautioned regarding future threats from the emerging resistance to antimicrobial agents of this not-so-common human pathogen. Similarly, different antimicrobial susceptibility profiles of *D. acidovorans* were also observed by Kawamura *et al*. (2011) wherein it was shown to be resistant to broad spectrum penicillins and cephalosporins but susceptible to ceftazidime, carbapenems, ciprofloxacin, minocycline, and trimethoprim–sulfamethoxazole. Khan *et al.* (2012) demonstrated the importance of proper identification of the Delftia species and their antibiotic resistance profile when an infection caused by an aminoglycoside resistant strain of *D. acidovorans* proved to be fatal. β-lactam resistance in *D. acidovorans* was found to be associated with β-lactamase production and alteration of outer membrane (OM) proteins (Ravaoarinoro and Therrien, 1999). Xu *et al*. (2007) characterized class 3 integrons from two environmental strains of *Delftia* spp. linking them to horizontal transfer of antibiotic resistance genes in the environment.

In recent times this bacterium has become a topic of interest when it was demonstrated that a secondary metabolite produced by *D. acidovorans* (delftibactin A) can protect against the toxic form of gold by binding and causing its biomineralisation (Johnston *et al.,* 2013). Another useful characteristic of *D. acidovorans* that has been well studied and exploited for environmental applications is its aniline-degrading capacity (Urata *et al.,* 2004). Aniline is a known environmental toxin and carcinogen that is used in the synthesis of herbicides and dyes and often released into the environment (Lyons *et al.,* 1985). Enzymatic degradation of xenobiotic compounds like

chlorinated phenoxyalkanoate herbicides (Yoon *et al.,* 2014) and resistance to high concentrations of these chemicals have also been demonstrated (Muller and Babel, 2004; Roland and Wolfgang, 2004). Biotransformation of selenium, lead and the toxic form of chromium ions by *Delftia* species have also been recently reported making them good candidates for cleaning up environmental pollutants (Ubalde *et al.,* 2012). A commercial biofertilizer formulation containing *D. acidovorans* is also available owing to its putative ability to oxidize sulfur (www. brettyoung.ca).

2.3. Chlorhexidine

According to the European Commission and European Chemicals Agency, 2014 "biocides are chemicals used to suppress organisms that are harmful to human or animal health, or that cause damage to natural or manufactured materials. These harmful organisms include pests and germs (i.e. moulds and bacteria). Examples of biocidal products are insecticides, disinfectants and industrial chemicals like anti-fouling paints for ships and material preservatives". Some types of biocides have been used for a century or more (e.g., alcohol) whereas others have been introduced only recently (orthophthaladehyde). Biocides have been used for clinical, pharmaceutical, cosmetic and preservation purposes. Among the popular biocides used in recent times is chlorhexidine. CHX is a broad-spectrum biocide used mainly in hospital, veterinary and household applications. CHX was initially developed by Imperial Chemical Industries, England, during the 1940's (Lindhe *et al.,* 2003). Later, in 1954, Davies *et al*. (1954) further modified the structure of the compound to improve its bactericidal properties. This resulted in 1,6 bis-4 chloro-phenlydiguanidohexane, a cationic bisbiguanide commonly referred to as CHX. It consists of two symmetric 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain. CHX is available as diacetate, gluconate and hydrochloride salts. It is more effective at alkaline pH and its activity is greatly reduced in the presence of organic matter (Russell and Path, 1986). The chemical structure of CHX is given below (**Figure 2.1**).

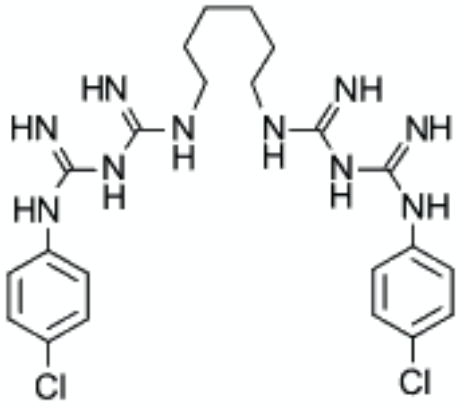

Figure 2.1 Structure of chlorhexidine (adapted from http://en.wikipedia.org/wiki/Image: Chlorhexidine.png)

Since the 1950's, CHX has been a popular, widely used biocide with a range of applications and is considered as the gold-standard antimicrobial agent against which other antimicrobial and antiplaque agents are assessed (Jones, 1997; Mathur *et al.,* 2011). CHX is presently an active ingredient in many commercially available disinfectants, antiseptics and pharmaceutical preservatives and is also listed in the World Health Organisation's list of essential medicines for basic health (http://www.who.int/medicines/ publications/ essentialmedicines/en/). It has found very wide applications as an antiplaque agent in oral hygiene products and is used extensively in dental therapy. CHX is commercially available at a variety of concentrations (0.5%–4%) and formulations and certain CHX-containing products are available over the counter (Milstone *et al.,* 2008). Based on their findings, Ugwumba *et al*. (2014) recommended the use of 0.2% CHX before dental extraction to reduce the risk of bacteraemia following dental extraction. Mouthwashes at 0.2% CHX also have a very effective antimicrobial effect on the salivary microflora (Addy *et al.,* 1991; Jenkins *et al.,* 1994) and dental plaque (Netuschil *et al.,* 1989). The American Heart Association (Dajani *et al.,* 1997) and the British Society for Antimicrobial Chemotherapy (Gould *et al.,* 2006) also recommend the use of CHX-containing mouthwashes at 0.2% to prevent dental infections. CHX usage at 0.12 % killed 98.5% of Gram negative non-fermentative bacteria in dental unit water lines (Kotaka *et al.,* 2012). Other non-dental applications include general skin cleansing, as a surgical scrub, gynecological antiseptic, wound disinfectant and as a pre-operative skin preparation. It is widely used as a preservative in eye formulations and contact lens solutions (Russell and Path, 1986). It is also found in chewing gums, toothpastes, varnishes and

periodontal dressings (Othman *et al.,* 1989; Smith *et al.,* 1995) and as teat disinfectants for use on dairy farms (Tremblay *et al.,* 2014). Landelle *et al*. (2014) recently reviewed the reduction of multidrug-resistant organisms (MRDO's) in intensive care units by CHX body washing of patients but also cautioned that vigilance is required for emerging CHX resistance. Edmiston *et al*. (2013) demonstrated that CHX is a very effective agent to inhibit or kill skin microflora and that a 1 min exposure to 0.05% CHX produced a > 5 log reduction in hospital associated pathogens and reduced biofilm formation on implantable medical devices.

CHX is bacteriostatic at low concentrations to many Gram positive and Gram negative bacteria and is bactericidal at higher concentrations, has antifungal properties, but is not sporicidal (Russell and Path, 1986). Despite its broad use, the exact mode of action of CHX remains unclear (Jones, 1997; Condell *et al.,* 2014). Nonetheless, like any other cationic antimicrobials, the general mechanism of action of CHX involves the bacterial cell membrane (Gilbert and Moore, 2005) and it is a potent membrane-active agent. CHX is a positively-charged, hydrophobic and lipophilic molecule and can interact with bacterial cell membrane components such as phospholipids and lipopolysaccharides. CHX possesses broad antibacterial and antifungal activity and seemingly exerts its effect by reacting with negatively-charged groups on the cell surface, altering the integrity of the bacterial cell membrane and activity of membrane-bound enzymes thereby causing an irreversible loss of cytoplasmic constituents, as well as membrane damage. At high concentrations (2%), CHX causes extensive cell damage, increasing the permeability of the inner membrane, coagulation of cytoplasmic constituents, and precipitation of proteins and nucleic acids (Hope and Wilson, 2004; Cheung *et al.,* 2012; Morente *et al.,* 2013). Several factors affect biocidal activity of CHX, most notably concentration, period of contact, pH, temperature, the presence of interfering materials, and the types, numbers, location and condition of microorganisms (Russell, 2003); however, optimal antimicrobial activity can be achieved within a pH range of 5.5-7.0 (Athanassiadis *et al.,* 2007). Morphological effects and structural damage of CHX on cell wall and cellular constituents have been reported by several research groups (Tattawasart *et al.,* 2000a,b; Shalamanov, 2005; Cheung *et al.,* 2012).

2.3.1. Chlorhexidine resistance in microorganisms

According to the Centers for Disease Control and Prevention, microbial resistance to antibiotics is perceived as representing an increasing threat to public health over the past several years. Some scientists suggest a similar situation exists with regard to the wide application of various biocides in daily use in households, hospitals, industry and the environment (Gilbert and McBain, 2003). Despite the use of many biocides for many years, there have been no reports of environmental outbreaks of such resistance; however, experts believe biocide resistance may eventually emerge on a large scale. Continuous overuse and resultant potential selective pressure could lead to increased tolerance to different classes of biocides and also more dramatically, the emergence of cross-resistance to various clinically-important antimicrobial compounds (Fraise, 2002; McBain *et al.,* 2002). However, several studies have demonstrated that there is no correlation between reduced susceptibility to biocides and resistance to clinically-relevant antimicrobial agents (Walsh *et al.,* 2003; Condell *et al.,* 2012). Though Thomas *et al.* (2000) demonstrated that repeated exposure to a sub-inhibitory concentration of CHX led to stable increases in the MICs of *P. aeruginosa* to chlorhexidine. An extensive study by Block and Furman (2002) involving different groups of organisms demonstrated an inverse correlation between the intensity of CHX use and the susceptibility of the organisms to CHX.

Resistance is the relative susceptibility of a microorganism to a particular treatment under a particular set of conditions. For antibacterial agents, it is commonly quantified in terms of the minimum concentration required to inhibit the growth of a population of cells, termed the MIC (Gilbert and McBain, 2003). On the other hand, a microorganism is said to be resistant to a biocide when it is not inactivated by an in-use concentration of a biocide, or a biocide concentration that inactivates other strains of the same organism (Russell, 2003). Although several microbes have been reported to survive in the presence of in-use concentrations of CHX (Marrie and Costerton, 1981; Stickler *et al.,* 1987; Brooks *et al.,* 2002), little is known about the underlying mechanisms for this tolerance. Resistance to biocides is often referred to as tolerance (Favero, 2002), since biocides usually have multiple targets and their efficacy is measured in terms of their rapidity to kill microorganisms. In simple terms, Cerf *et al*. (2010)

proposed that the terms “resistance” should be used when the phenomenon being studied is killing and “tolerance” when it is used in reference to adaptation to inhibitory concentrations. There is no fixed minimum inhibitory concentration (MIC) value for CHX. It varies depending on the type of bacterial strain, phenotypic and genotypic status of the organism and the environmental conditions. Walker and Lowes (1985) found that MIC testing was unreliable in the assessment of CHX resistance as it was dependent on the medium used, the age of the culture, and size of inoculum indicating that CHX resistance is a complex phenomenon which is difficult to evaluate. Russell and Day (1993) indicated that as a general rule, CHX inhibits Gram positive and Gram negative bacteria at concentrations of 1 μg ml-1 and 2-2.5 μg ml-1, respectively.

MIC values of a *P. aeruginosa* strain recommended for disinfectant testing and an industrial strain were found to be 60-85 μg ml^{-1} and 35-45 μg ml^{-1}, respectively (Lear *et al.,* 2006). The MIC for CHX of many of the strains isolated from the skin of patients undergoing intermittent bladder catherization proved to be 200-800 μg ml^{-1} compared with the 10-50 μg ml^{-1} level recorded for reference strains of Gram negative bacteria (Stickler, 2002). In a recent study (Kishk *et al.,* 2014), the MIC’s of 1% CHX for 56 *Klebsiella pneumoniae* strains isolated from human blood specimens ranged from 4 to 256 mg l^{-1}. Koljalg *et al*. (2002) conducted CHX susceptibility testing of 70 distinct clinical isolates including *Pseudomonas spp.* and found that non-fermentative bacteria tolerated CHX at higher concentrations and Gram positive cocci, especially *Streptococcus pyogenes*, were the most susceptible. Gram negative bacteria had a wider range of MIC values (1-64 mg l^{-1}) than Gram positive bacteria (0.25-8 mg l^{-1}). *Pseudomonas stutzeri* and *P. aeruginosa* became increasingly resistant to CHX when exposed to gradually-increasing concentrations of the biguanide. The MIC’s increased from 2.5 to 50 mg l^{-1} and 10 to 100 mg l^{-1}, respectively, within 12 days (Tattawasart *et al.,* 1999). Similarly, Stickler and Thomas (1980) found that amongst the Gram negative isolates obtained from urinary tract infections, those belonging to the genera *Proteus*, *Pseudomonas* and *Providencia* were predominantly resistant to CHX at concentrations of more than 500 μg ml^{-1}; whereas, most triclosan and *para*-chloro-*meta*-xylenol-tolerant strains, including *P.* aeruginosa and *P. stutzeri,* did not show increased resistance to other biocides like CHX, benzalkonium chloride (BAC) and phenol (Lear *et al.,*

2006). On the other hand, screening of 472 clinical isolates and 693 stock cultures that included several species and also antibiotic resistant strains, found a low incidence of CHX resistance and no isolates exhibited high-level CHX resistance (Barry *et al.,* 1999). Almost 98.5% of Gram negative non-fermentative rods isolated from dental unit water lines were killed by 0.12% CHX, which included 100% of all the *D. acidovorans* strains (Kotaka *et al.,* 2012). Very recently, an isolate identified as *Aeromonas hydrophila* was found to grow in 0.02% CHX in a natural water source in Kansas City, giving rise to a new healthcare concern. This strain was found to be resistant to various other antibiotics, including vancomycin, ampicillin, ciprofloxacin and tetracycline (Sekavec *et al.,* 2013). There are mixed reports either agreeing (Lamfon *et al.,* 2004; da Silva *et al.,* 2014) or disagreeing (Bonez *et al.,* 2013) with the commonly-held paradigm that biofilm cells exhibit greater resistance to antimicrobials than their planktonic counterparts when CHX activity was specifically evaluated. CHX was shown to be effective against biofilms of some microorganisms but not others; for example, *Candida albicans* biofilms were destroyed by MIC levels of CHX for planktonic cells, it was not the same situation in the case of *P. aeruginosa* and *Acinetobacter baumannii* biofilms. Thus, the composition of biofilms and penetration of CHX through the cell wall may influence the activity of CHX (Bonez *et al.,* 2013).

2.3.2. Effect of chlorhexidine on microbial biofilms

Bacterial cells growing on surfaces as biofilms are in general protected from different kinds of environmental stressors, including antimicrobials. The majority of work conducted on CHX has thus far involved dental biofilms whereas relatively little research has examined pure culture biofilms. In general, pure culture studies have shown that CHX affects the viability of biofilm cells, the live dead ratio, biofilm structure and thickness. Hope and Wilson (2004) observed the contraction of biofilms when exposed to 0.2% CHX, as well as time-dependent shifts in viability profiles through multispecies biofilms. In a study of the tongue biofilm microflora, Sreenivasan and Gittins (2004) reported that a 0.12% CHX mouth rinse resulted in a 90, 85, 90, 94 and 81% decrease of anaerobic, Gram positive, Gram negative, H_2S producing and proteolytic bacteria, respectively.

CHX treatment at a concentration of 0.2% resulted in greater reductions in viable counts and organic acid formation in polyacrylic grooves than in dentin grooves by *Streptococcus mutans* biofilms (Deng *et al.,* 2004). Wilson *et al*. (1998) showed that biofilms grown in the presence of sucrose were more susceptible to CHX than those grown in the absence of sucrose, and that the dominant microflora of these two biofilm types varied. In addition, substantial numbers of bacteria remained viable even after exposure to 0.2% CHX for 60 min. Thus, CHX has potential as a selective agent, preferentially killing specific populations within a community as well as selecting for resistant community members. Using CLSM, Tremblay *et al*. (2014) showed that 0.525% CHX could kill biofilms formed by coagulase-negative staphylococci isolated from milking dairy cows as opposed to a lower level (0.001%) of CHX (Chiang *et al.,* 2012) which killed only the inactive subpopulation of *P. aeruginosa* cells located in the deep layers of the biofilms leaving an active subpopulation located in the upper layer of the biofilm. The active subpopulation was able to tolerate CHX due to the use of some active genetic determinants in this case the mexCD-oprJ genes. CHX at MIC levels (1.5 μg ml^{-1}) promoted biofilm detachment and inhibited detachment when concentrations were higher than the MIC and reduced detached-cell viability only at high concentrations (1.6 times the MIC) in 48 h old *Streptococcus mutans* biofilms formed on polystyrene blocks (Liu *et al.,* 2012). In this study, the effects of CHX on biofilm detachment differed depending on treatment time and concentration. The proportion of killing of bacteria in biofilms by CHX can also depend on the age of the biofilm (Shen *et al.,* 2011). A TEM and SEM study revealed that 0.1% CHX did not induce ultra-structural alterations in an oral biofilm exposed to the biocide for 1 min although membrane damage and blebbing was observed in one third of the cells after a 5 min exposure at the same concentarion (Vitkov *et al.,* 2005). Similarly, the prolonged exposure of *S. mutans* biofilms to sub-minimal bactericidal (4.5 μg ml^{-1}) concentrations of CHX caused membrane rupture and loss of cytoplasmic contents while high concentrations (2000 μg ml^{-1}) of the compound caused extensive precipitation of an unknown material on the slides (data not shown) (da Silva *et al.,* 2014).

2.4. Antimicrobial resistance mechanisms in biofilms

Antimicrobial resistance in biofilms is one of the most-studied topics in biofilm biology although there is no straightforward, or single, answer as to why biofilm microbial cells generally exhibit greater antimicrobial resistance compared to their planktonic counterparts. Indeed, the fundamental physical, chemical and biological mechanisms by which biofilm bacteria resist the killing effect of biocides and antibiotics remain incompletely understood. An examination of the literature indicates that antimicrobial biofilm resistance is a result of several factors that may act in combination and can vary from structural components of individual cells (e.g., outer membrane) to the biofilm structure itself (slow penetration through a thick biofilm) as well as the involvement of molecular level processes (Mah and Toole, 2001; Stewart and Costerton, 2001; Hoiby *et al.,* 2010a; Mah, 2012). Russell (1995) summarised the mechanisms of bacterial resistance or tolerance to biocides and indicated that they could be either intrinsic, acquired, or both. Intrinsic resistance is defined as a natural chromosomally-controlled property of a bacterial cell that enables it to survive biocidal action and may vary between different strains of the same species. In terms of intrinsic resistance among vegetative bacteria, mycobacteria are probably the most resistant, followed by Gram negative bacteria, with the most sensitive being the Gram positive bacteria. Examples of intrinsic resistance traits include composition of outer cell layers, spore formation, phenotypic adaptation including biofilm formation and production of degradative enzymes (Morente *et al.,* 2013). Acquired resistance results from genetic changes in a bacterial cell either through mutation or by acquisition of extra-chromosomal elements such as plasmids or transposons. Exposure to gradually increasing concentrations of biocide may also result in acquired resistance (Fitzgerald *et al.,* 1992; Mangalappalli-Illathu and Korber, 2006). Similarly, permeability changes either through ultrastructural, compositional or hydrophobicity changes in the bacterial cell envelope may lead to acquired resistance, especially in Gram negative bacteria (Morente *et al.,* 2013). Based on available data, Poole (2002) summarised that general tolerance to biocides, including CHX, typically does not develop following mutation of a particular target gene but rather involves broader cellular changes, such as up-regulated efflux pump activity, alterations in cell wall permeability, and changes in fatty acid profiles.

In an earlier study, Suci *et al*. (1994) demonstrated that transport limitation could be an important factor in the antimicrobial resistance of biofilm bacteria; however, Mah *et al*. (2003) reported that biofilms themselves may not act as a diffusion barrier to antibiotics but the bacterial cells within these biofilms may have some distinct resistance mechanisms to combat the action of antimicrobials. To prove this, they showed that the periplasmic glucans produced by *ndvB* contributed to antibiotic resistance in biofilms by sequestering the antibiotics and preventing them from reaching their cellular targets. Zhang *et al*. (2013) suggested that when two or more genes that are not functionally-related are responsible for antibiotic resistance, multiple biochemical pathways need to be considered as contributors for this trait. Only the major antimicrobial resistance mechanisms will be discussed in detail in this section, in particular, those that are deemed relevant to this thesis, and where available, those that are applicable to CHX. The mechanisms associated with biofilm resistance to CHX have received varying degrees of attention and the literature consists of largely phenomenological descriptions of its occurrence rather than in-depth studies of the actual mechanisms involved.

Some bacterial genera (e.g., Proteus, Providencia) may either be intrinsically-resistant to CHX or have inducible mechanisms of resistance due to phenotypic and genetic changes. Protection against antimicrobial agents could also be mediated through the existence of sub-populations of resistant cell phenotypes in the biofilm known as 'persisters' (Stoodley *et al.,* 2004; Keren *et al.,* 2004). Russell and Day (1993) indicated in their review that the genetic basis of CHX resistance may be due to transfer by recombinant plasmids (as seen in *S. aureus*), by genetic transformation (*Streptococcus sanguis*), or through chromosomally mediated mechanisms. The authors added that one of the reasons for high levels of CHX resistance may be associated with the barrier properties presented by the outer membrane of organisms, as well as the existence of microorganisms in the form of biofilms. In general, a single target site or point-mutations, are rare in the case of resistance to biocides due to their cell-wide, non-specific modes of action (Russell, 2003).

2.5. Chlorhexidine resistance in biofilms

It is commonly reported that bacterial biofilms may be up to 1,000 times more resistant than their planktonic counterparts (Nickel *et al.,* 1985; Tremblay *et al.,* 2014). Several mechanisms have been proposed to explain the general of biofilms to biocidal agents, the most common of which are the barrier properties of the 'slime' matrix or reduction in access via reaction-diffusion kinetic limitations imposed by biofilm components (Chapman, 2003). A study by Fitzgerald *et al*. (1992) showed that the uptake of ^{14}C-CHX by strains of *P. aeruginosa* and *E. coli* was very rapid (within 20 s). Despite the rapid uptake, the lethal action of CHX was comparatively slow. Their study further determined that penetration failure is the most common resistance mechanism when dealing with thick biofilms and highly- reactive antimicrobials such as CHX. In contrast, studies using ciprofloxacin and tobramycin in *P. aeruginosa* biofilms indicated that limited antibiotic diffusion was not the primary protective mechanism for resistance in these biofilms. Rather, protection was mediated via oxygen limitation and low metabolic activity in the interior of the biofilm (Walters *et al.,* 2003). Similar conclusions were made in another study (Anderl *et al.,* 2000) wherein antibiotic inactivation or slow diffusion were not the mechanisms of resistance to ampicillin and ciprofloxacin in *Klebsiella pneumoniae* biofilms. The slow growth of microorganisms within biofilms reduces their susceptibility to biocides by a number of factors that include nutrient depletion within the biofilm, chemical interaction between the biocide and biofilm cells, and production of neutralizing enzymes and chemicals (Gilbert and Moore, 2005).

2.5.1. Chlorhexidine resistance by microbial/enzymatic degradation

Enzymatic resistance can be achieved either by destroying or by modifying the antimicrobial compound. The chemical strategies of antibiotic inactivation include hydrolysis, group transfer, and redox mechanisms (Wright, 2005). Examples include the enzyme β-lactamase produced by *P. aeruginosa* which inactivates many β-

lactam antibiotics by β-lactam ring cleavage (Bagge *et al.,* 2004). Acetylating or adenylating enzymes produced by some Gram negative bacilli cause increased resistance to gentamicin (Dornbusch and Hallander, 1980). CHX degradation by activated sludge communities has also been reported (Sayama, 1981; Sakagami *et al.,* 1983). Tanaka *et al*. (2005) reported that a *Pseudomonas* sp. strain degraded CHX to a number of transient intermediate compounds which were identified using HPLC methods. Results indicated that two pathways were involved in this degradation process. Interestingly, the MIC values for the degradation end products like CHDI-B ($C_{25}H_{34}C_{l2}N_{10}O_2$) and CHDI-C ($C_{24}H_{32}N_{10}C_{l2}$) were found to be 5 to 10 times higher than for CHX itself. Other CHX end products, such as *p*-chlorphenylurea and *p*-chloraniline, had no antimicrobial activity (Tanaka *et al.,* 2005). CHX degradation by clinical isolates of *P. aeruginosa*, *P. cepacia*, *Alcaligenes faecalis*, *Alcaligenes xylosoxidans*, *Serratia marcescens* and *Pseudomonas* sp. strain A-3 (as a control CHX-degrading organism) have also been investigated (Uyeda *et al.,* 1996). Among these organisms, only *S. marcescens* showed similar degradation, via a modified pathway, as the control organism. A *Pseudomonas* sp. and a *Flavobacterium* sp. showed different patterns of CHX degradation, wherein the former yielded transient intermediates and the latter did not (Kido *et al.,* 1988). Strains of *P. aeruginosa* converted CHX to *p*-chloroaniline, *p*-chlorophenol, *p*-chloroacetoanilide, phenol, aniline, pyrocatechol and pyrogallol (Sakagami *et al.,* 1986). Hence, degradation can be considered as one of the potential mechanisms for resistance to CHX by some bacteria.

2.5.2. Role of the outer membrane in chlorhexidine resistance

Several authors have focused on the role of the outer membrane as a physical barrier to the effects of antibiotics, disinfectants and detergents, especially in Gram negative bacteria (Hancock, 1997; Dykes *et al.,* 2003; Sampathkumar *et al.,* 2004; Ruiz *et al.,* 2006). The OM separates the periplasm of bacterial cells from the external environment, and owing to its physical properties, acts as a selective barrier, preventing the entry of many toxic molecules into the interior of the cell, a property that is considered crucial for survival in many environments. Similar speculation suggesting that the OM plays a key role has been made regarding the membrane-active agent

CHX (Richards and Cavill, 1979; Russell and Path, 1986; Kuyyakanond and Quesnel, 1992; Tattawasart *et al.,* 2000a, b; Maillard, 2002; Castillo *et al.,* 2006). Cationic antimicrobials such as CHX bind to the negatively-charged lipopolysaccharide (LPS) of the outer membrane and subsequent entry into the cells depends on the transmembrane electrical potential (Damper and Epstein, 1981; Bryan and Kwan, 1983). Any change in the LPS can increase or decrease the binding affinity of the LPS for polycations, thus making the cells more or less susceptible to these antimicrobials (Rivera *et al.,* 1988). Divalent cations like calcium and magnesium maintain the integrity of the OM, and thus the presence of a chelator-like EDTA can disrupt the OM, increasing the susceptibility to hydrophobic compounds (Ayres *et al.,* 1998; Russell, 2003; Ruiz *et al.,* 2006) and antibiotics (Hancock, 1997). Overall changes in the protein content (Gandhi *et al.,* 1993) and fatty acid composition (Guérin-Méchin, 1999; Méchin *et al.,* 1999) have also been shown to influence the susceptibility of Gram negative bacteria to antimicrobial agents. Adaptation of *S. marcescens* to 0.006% CHX was consistent with alterations in the outer membrane proteins (Gandhi *et al.,* 1993). *Pseudomonas aeruginosa* cells adapted to a quaternary ammonium compound (QAC) showed variations in membrane fatty acid composition with significant involvement of five fatty acids, namely lauric acid (C12 : 0), β-hydroxycapric acid (C10 : 0 β-OH), α- and β-hydroxylauric acids (C12 : 0 α- and β-OH), and palmitoleic acid (C16 : 1 cis 9) (Guérin-Méchin *et al.,* 1999). The total lipid content of *C. albicans* grown in the presence of a sub-inhibitory concentration of CHX declined while the total sterol content was increased compared to control cells. CHX-exposed yeasts had a higher level of phosphatidylethanolamine, phosphatidylcholine and monogalactosyldiacylglycerol and lower proportions of phosphatidylinositol, phosphatidylserine, phosphatidic acid, cardiolipin and higher proportions of palmitic acid (16: 0) and stearic acid (18: 0), but lower proportions of palmitoleic acid (16: 1) and oleic acid (18: 1). CHX also decreased the unsaturated-to-saturated fatty acid ratio, while the C16/C18 ratios increased compared to control cells (Abu-Elteen and Whittaker, 1997).

The presence of porins and negatively charged LPS in combination with an active efflux system is probably responsible for the high resistance of the Gram negative OM to externally added detergents and dyes (Hancock, 1997). However, Gilbert and Moore (2005) reported that resistance to CHX was based on action at the OM and

that multi-drug efflux pumps did not play a role, presumably because the bisbiguanide is not soluble within the membrane core. Russell (2003) also indicated that resistance to CHX by Gram negative organisms like *P. aeruginosa* is due to reduced uptake of the biocide owing to an impermeable cell membrane. Differences in the OM and release of cell contents were observed in CHX-treated *P. aeruginosa* and *P. cepacia* cells at CHX concentrations of 10.0 and 3.0 μg ml^{-1}, respectively (Richards and Cavill, 1981). A previous study by the same group (Richards and Cavill, 1979) showed that low levels of CHX (2.0-3.0 μg ml^{-1}) acted primarily on the cytoplasmic membrane and on the cytoplasmic membrane plus layers external to it at concentrations greater than 3.0 μg ml^{-1}. The *opr*D, E, L and F genes that encode OM proteins for maintaining the integrity of the OM and control of OM permeability in *P. aeruginosa* were found to be down-regulated after 60 min of CHX exposure, indicating that CHX treatment affected OM permeability (Nde *et al.,* 2009). The presence of an anion-selective porin from *Comamonas acidovorans*, Omp32, has been reported and may have biological implications in terms of strong selectivity and specificity for extra-cellular molecules (Zeth *et al.,* 2000).

Transmission electron microscopy (TEM), scanning electron microscopy (SEM) and energy dispersive X-ray analysis (EDAX) have been used to examine the effects of chlorhexidine diacetate (CHA) on CHA-sensitive and CHA-resistant isolates of *P. stutzeri*. CHA caused structural damage and extensive lysis to CHA-sensitive cells; whereas, no such effect was seen on CHA-resistant cells. The results of X-ray mapping confirmed the difference in CHA uptake between these two groups of isolates. The concentration and distribution of magnesium and calcium (both involved in the structural organization of the outer membrane), phosphorous (structural element) and chlorine (marker for CHA) were studied and the results led the authors to conclude that the major mechanism of CHA resistance in *P. stutzeri* may be linked to changes in the binding sites available in the outer membrane. They speculated that deletion or depression of a porin protein resulted in the decrease in cell permeability (Tattawasart *et al.,* 2000a,b).

2.5.3. Role of efflux pumps in chlorhexidine resistance

The role of energy-driven drug efflux systems as a mechanism of antibiotic resistance has been extensively studied and reviewed (Lomovskaya *et al.,* 2001; Poole, 2001; Levy, 2002). Efflux pumps are transporter proteins that extrude toxic substances from within the cells into the external environment by being specific to one substrate or a range of structurally-similar compounds, in the latter case leading to multidrug resistance. Efflux pumps are found in both Gram positive and Gram negative bacteria. It has been estimated that approximately 5–10% of all bacterial genes are involved in trans-membrane transport and a large proportion of these genes encode efflux pumps (Webber and Piddock, 2003). Bacterial efflux pumps can be broadly divided into five classes: the small multidrug resistance (SMR) family, the major facilitator superfamily (MFS), the ATP-binding cassette (ABC) family, the resistance-nodulation-division (RND) family and the multidrug and toxic compound extrusion (MATE) family (Webber and Piddock, 2003).

Efflux proteins in *P. aeruginosa* have been widely studied and shown to be associated with resistance to antibiotics and biocides, including CHX. Sequencing of the *P. aeruginosa* genome has revealed at least 12 efflux pumps, of which the MexAB-OprM pump is the most extensively characterized and most commonly-expressed in wild type strains. This efflux pump has been found to act on a range of antibiotics and dyes (Srikumar *et al.,* 1998), including cationic agents like aminoglycosides (Li *et al.,* 2003). Chromosomally-located or acquired by bacteria, efflux pumps can either be activated by environmental signals or by a mutation in a regulatory gene. One such pump, the AcrAB pump, extrudes biocides such as triclosan, CHX and quaternary ammonium compounds, as well as multiple antibiotics. The multidrug efflux operon mexCD-oprJ was found to be induced by CHX in *P. aeruginosa* (Morita *et al.,* 2003; Fraud *et al.,* 2008; Nde *et al.,* 2009) and was found to be dependent on the stress response sigma factor, AlgU (Fraud *et al.,* 2008), which is homologous to RpoE in *E. coli*. CepA, a cation efflux pump, is associated with CHX resistance in *K. pneumoniae* and possibly in other Gram negative bacteria (Fang *et al.,* 2002; Kishk *et al.,* 2014). The RND efflux system may be primarily responsible for CHX resistance in Gram

negative bacteria such as *P. aeruginosa* (Nde *et al.,* 2009) and *Burkholderia cenocepacia* (Coenye *et al.,* 2011); whereas, the AdeABC efflux system has been reported in clinical *A. baumannii* isolates (Rajamohan *et al.,* 2010; Hassana *et al.,* 2013). Association of the qacA/B efflux gene and CHX tolerance or has been demonstrated in several studies in *S. aureus* (Smith *et al.,* 2008; Wang *et al.,* 2008).

Several studies have concluded that although the OM of *P. aeruginosa* has very low non-specific permeability, this alone is not sufficient to explain their high resistance to a wide variety of toxic molecules and that interplay between OM and multidrug efflux pumps would be required. Efflux pumps work synergistically with the outer membrane. The OM itself does not provide resistance to antibiotics as it only has decreased permeability. Studies on the interplay between efflux pumps and the OM in cases of multiple antibiotic resistance have been conducted in *P. aeruginosa;* however, these studies have been restricted to planktonic cell cultures. No such study has been reported in biofilms. Inactivation of MexA-MexB-OprM multidrug efflux pump and increasing the OM permeability by use of permeabilizers such as EDTA and NaHMP have been shown to enhance the antibiotic susceptibility in *P. aeruginosa* (Li *et al.,* 2000). Similarly, Germ *et al.* (1999) demonstrated the interplay between MexAB-OprM and the OM permeability barrier in lowering the intracellular concentration of fluorescent dyes in *P. aeruginosa*. In this case, they used EDTA (0.1 mM) and polymyxin B (2.5 $\mu g\ ml^{-1}$) to enhance the permeability of the OM.

2.5.4. Role of persister cells in CHX resistance

In 1944, Joseph Bigger discovered a sub-population of cells within a Staphylococcus culture that survived repeated exposures to penicillin and differentiated them from resistant mutants as persisters. Recently, Zhang (2014) summarized the drawbacks of previous definitions of persister cells, and proposed a Yin-Yang model to better describe this phenomenon. His new definition is as follows; “persisters refer to genetically-drug-susceptible quiescent (non-growing or slow growing) organisms that survive exposure to a given antibiotic or drug and have the capacity to revive (regrow or resuscitate and grow) under highly-specific conditions”.

The persister cell theory has gained serious attention in recent times as it is now believed that these dormant variants are most likely responsible for the recalcitrance of chronic infectious diseases to antimicrobial treatments and can promote multidrug tolerance. It is quite challenging to use the existing antibiotics effectively against dormant persister organisms. The principal role of persisters in biofilm antimicrobial tolerance and chronic infections have been reported and reviewed extensively (Harrison *et al.,* 2005; Lewis *et al.,* 2005; LaFleur *et al.,* 2006; Mulcahy *et al.,* 2010). LaFleur *et al.* (2006) demonstrated the existence of persisters in *C. albicans* biofilms treated with CHX and that they were not mutants but phenotypic variants of the wild type. They also concluded that effluxers do not contribute to persister survival and CHX resistance in *C. albicans* biofilms. Furthermore, quorum sensing did not seem to play a role in persister formation in *E. coli* and persister numbers increased in the mid-exponential phase, reaching a maximum of ~1% persisters in the stationary phase (Lewis, 2007).

It is now known that several genes and pathways are involved in persister formation and survival and this is discussed in detail later in the proteomic section of this chapter. Generally, persister genes belong to the SOS stress response systems, *hipA* being the first *bonafide* persister gene (Keren *et al.,* 2004). Some of the multidrug tolerant loci found in *E. coli* persisters are hipBA toxin/antitoxin loci (Keren *et al.,* 2004), sn-glycerol-3-phosphate dehydrogenase GlpD and plsB, and sn-glycerol-3-phosphate acyltransferase (Spoering *et al.,* 2006).

2.6. Microscopic analysis of microbial biofilms

Microscopic examination of biofilms began in the days of Antonie van Leeuwenhoek (1684) and has naturally undergone considerable advancements since then. Developments in microscopy have enabled researchers to develop a better understanding of biofilm structure and function, as well as to study their interactions with various antimicrobials (Vitkov *et al.,* 2005; Mangalappalli-Illathu and Korber, 2006; Dynes *et al.,* 2009; Neu *et al.,* 2010; Shen *et al.,* 2011; Tremblay *et al.,* 2014). Microscope-based techniques such as confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM) and scanning transmission X-ray microscopy

(STXM) have been used singly, or in combination, to examine complex microbial communities as well as to map the extent, nature and distribution of macromolecules in a biofilm (Lawrence *et al.,* 2003; Dynes *et al.,* 2009). Each technique has its own advantages and disadvantages and can be selected based on the application and objectives to be achieved (Neu *et al.,* 2010; Pantanella *et al.,* 2013).

Confocal laser scanning microscopy is a non-invasive, non-destructive method that may be used to monitor biofilms *in situ*. It is a powerful technique that not only allows researchers to take images with microscopic resolution, but also reveals the system's three-dimensional structure. Cell viability, cell morphology, biofilm architecture and matrix composition can be studied in living, fully-hydrated systems using CLSM (Korber *et al.,* 1994; Lawrence *et al.,* 2003; Shen *et al.,* 2011). Multi-wavelength/channel visualization of the biofilms is one of the greatest advantages of using this technique. Microbial communities can be identified using CLSM and rRNA-targeted oligonucleotide probes using the fluorescence *in situ* hybridization technique and structure-function relationships can be also be studied using fluorescent reporter genes such as green fluorescent protein (GFP) (Neu *et al.,* 2010). Many authors have used CLSM to visualize the three-dimensional structure of biofilms and compared this approach to other microscopic techniques such as electron microscopy. Manz *et al*. (1999) employed CLSM to study the three-dimensional structure and dynamics of bacterial communities in river biofilms generated using a rotating annular reactor system. Eaglesham *et al*. (2003) used CLSM to observe the channels, pores, and other structural features of a biofilm matrix grown in lake water. Similarly, many other research groups have employed CLSM for biofilm studies (Battin *et al.,* 2003; Teitzel and Parsek, 2003; Hope and Wilson, 2004; Lawrence *et al.,* 2005; Shen *et al.,* 2011) in conjunction with vital staining or fluorescent probing techniques. Fluorescent dyes such as SYTO9 and propidium iodide can be employed in combination with CLSM to evaluate the potential for antimicrobial agents to influence membrane integrity and permeability (Dynes *et al.,* 2009; Tremblay *et al.,* 2014). Hope and Wilson (2004) concluded in their study that fluorescent indicators can readily be used to investigate the penetration and antimicrobial effects of membrane active biocides like CHX on biofilms. Because it is known that CHX damages the bacterial membranes, its bactericidal action can be measured in real time using probes like the BacLight LIVE/DEAD nucleic acid stain. This stain, which is sensitive to the condition of the cell's cytoplasmic

membrane, has been used by a number of other research groups for viability studies in biofilms. Decker *et al.* (2003) used these stains for susceptibility studies of *Streptococci sanguinis* cells to CHX. The morphology and viability of Staphylococci biofilms exposed to CHX was examined using CLSM and staining techniques (Tremblay *et al.,* 2014). The observations included reduction in the amount of bacteria and presence of a fairly large amount of permeabilised cells, as indicated by red cells after CHX treatment. Vitality distribution in CHX-treated biofilms is dependent on a number of factors, such as the substrate on which the biofilms have been formed, treatment of the biofilms before imaging (intact hydrated versus dried and fixed biofilms) and unclear distinction between red and green ("dead" and "living", respectively) cells due to the presence of an intermediate orange color (Zaura-Arite *et al.,* 2001). Measurements such as biofilm thickness, percentage of live cells, and distribution of live cells at various depths have been obtained using CLSM to study the bactericidal effect of CHX treatment on undisturbed plaque biofilms grown on bovine dentin discs (Zaura-Arite *et al.,* 2001). Similarly, Shen *et al*. (2011) used CLSM and Live/Dead vitality stain to study antimicrobial efficacy of CHX against bacteria at different stages of development and concluded that CHX resistance (in terms of proportion of dead cells) was dependant on the age of the biofilm. Chiang *et al*. (2012) used CLSM in combination with GFP markers to study the molecular determinants of CHX resistance in *P. aeruginosa* biofilms. Using propidium iodide staining they showed that CHX at 0.001% killed the inactive subpopulation located deep in *P. aeruginosa* biofilms; whereas, the active subpopulation located in the upper layer of *P. aeruginosa* biofilms survived the biocide treatment. In addition, using this technique they also demonstrated that development of CHX-tolerance was dependent on the presence of *mexCD-oprJ* genes, but did not depend on the *pmr*, *mexAB-oprM*, *mexPQ-opmE*, or *muxABC-opmB* genes.

The distribution and concentration of CHX in combination with biomacromolecules (e.g., proteins, lipids, polysaccharides) can further be investigated using STXM at a spatial resolution of <30 nm in hydrated biofilm systems (Dynes *et al.,* 2006a, 2009). STXM was first developed by Kirz and Rarback (1985). Based on the differences in the elemental composition and bonding structure of a particular chemical species, the STXM approach uses the near edge X-ray absorption spectrum (NEXAFS) (Stohr, 1992) so that the chemical state and the morphology of the sample can be directly correlated (Bluhm *et al.,* 2006). Both soft and hard X-rays can be used

for this purpose since most elements have an absorption edge in both energy ranges; however, soft X-rays are preferentially-used over hard X-rays to collect C, N and O information for quantitative mapping of organic biomolecules in biofilms. STXM at the C 1s edge has been exploited in several studies for mapping the major biomacromolecules in a biofilm (Lawrence *et al.,* 2003, 2012; Dynes *et al.,* 2009; Liu *et al.,* 2013; Rema *et al.,* 2014). Sequences of images are recorded over a span of photon energies at core excitation edges by scanning the sample through the focal point of a zone plate X-ray lens and measuring the intensity of transmitted X-rays. This data is converted to quantitative maps of these compounds using suitable quantitative reference spectra. The sequence of images collected at a range of different energies is referred to as an image "stack". Using this principle, synchrotron x-ray microscopy can be used to obtain a detailed mapping of biofilm structure and to understand the biochemical basis for biofilm formation. STXM is ideally suited to investigate small particles, membranes and whole bacteria immersed in aqueous solution with minimal sample preparation since soft X-rays can penetrate water; however, the sample thickness can be a limiting factor (soft x-rays penetrate poorly through thick biofilms). Lawrence *et al*. (2003) carried out correlative mapping studies of structure, composition and distribution of macromolecular components in a river biofilm system using TEM, CLSM, and soft X-ray scanning transmission microscopy (STXM). Such correlative approaches with synchrotron-based and other microspectroscopic and imaging techniques will allow a better understanding of both the fate and effects of environmental contaminants and chemicals when interacting with microbial biofilms. It is believed that STXM data may be better than TEM owing to its higher energy resolution and reduced photon damage of the sample (Wang *et al.,* 2009). However, spectral fitting of organic contaminants for proper distinction against organic biomolecules in a microbial biofilm can be challenging and may warrant a different approach for such studies. Obst *et al*. (2009) used STXM to study calcium precipitation by cyanobacteria and the role of EPS in this process. They were successful in investigating the earlier stages of cyanobacterial biomineralisation which was not possible by other techniques such as Raman spectroscopy, CLSM or TEM because of the small spatial scale involved and also due to the limited speciation capability and production of artifacts in the sample leading to underestimation of the biological components, particularly the EPS. Dynes *et al*. (2006a) employed STXM to investigate the speciation

and distribution of metals in a river microbial biofilm. An additional study (Dynes *et al*. 2006b) demonstrated the mapping of CHX in microbial biofilms and demonstrated its association with cellular lipids. Three-dimensional imaging of samples using STXM was also achieved with ~50 nm spatial resolution (Johansson *et al.,* 2007). Therefore, this imaging approach has considerable potential in studies of fate and effects of CHX in microbial biofilms.

Similar to STXM, infra-red (IR) spectroscopy is a non-destructive technique that requires minimal sample preparation to study biological materials wherein the IR portion of the spectrum is used to study the vibrational and rotational motions of atoms in molecules. Vibrational spectroscopic methods are based on the absorption (Fourier transformed infrared spectroscopy-FTIR) or scattering (Raman microscopy) of light directed onto a sample. The IR spectrum is obtained by determining the fraction of incident radiation that is absorbed by the sample at a particular energy and each peak in the spectrum corresponds to the vibration of a part of the sample molecule. In FTIR microspectroscopy, every pixel in an image corresponds to a complete IR spectrum that in turn correlates to the chemical composition of the analysed spot. Common biomolecules, such as nucleic acids, carbohydrates, proteins and lipids, have characteristic vibrational modes that are used in microbial and ecological studies. Thus, vibrational signatures of chemical bonds in biomolecules within a biofilm with a spatial resolution of <10 μm can be obtained using synchrotron radiation-based Fourier transformed infrared spectroscopy (SR-FTIR). Most of the relevant biological information can be obtained from the mid-infrared region (4000-800 cm^{-1}) (Suci *et al.,* 1998). Different covalent bonds have specific IR absorption peaks that change based on structural/bonding modifications and it is this principle that is taken advantage of while investigating the environmental influence on biological samples such as biofilms. SR-FTIR microscopy offers good signal to noise ratio and wave number precision (Griffiths, 1983) and can be used to track biogeochemical changes within a biological sample with high sensitivity and micrometer spatial resolution in real time (Holman *et al.,* 2009b) with a power below the threshold for cytotoxic effects (Holman *et al.,* 2002). Infrared absorption spectra of organic molecules provide unique "fingerprints" that can be applied for identification purposes of microbes with great reliability (Suci *et al.,* 1998). Strong amide I (between 1690 and 1650 cm^{-1}) and amide II (1550 cm^{-1}) versus an enlarged O-H and carbonyl peak at 1740 cm^{-1} helped to

differentiate between two groups of bacteria, *E. coli* from *Bacillus subtilis* cells, in an extensive study reported by Nichols *et al*. (1985). In this study, the authors were also able to recognise the differences in microbial community structure by subtracting the spectra obtained for these two groups of bacteria. Differences in the band pattern were also seen in bacterial polymers such as gum arabic and poly-β-hydroxybutyrate (PHB). Bacterial biofilm changes that occurred during: i) adaptation to their surrounding environments, ii) transportation, adsorption and interaction kinetics of antimicrobial agents within the biofilms (Suci *et al.,* 1994; Suci *et al.,* 2001; Holman *et al.,* 2009a); Rema *et al.,* 2014) and iii) biofilm developmental stages (Quiles *et al.,* 2010) have also been monitored using SR-FTIR. Reuben *et al*. (2014) combined CLSM with SR-FTIR imaging to better understand the spatial chemical composition of multispecies biofilms grown from natural storm waters in flow cells especially with respect to high density and void regions of the biofilm.

2.7. Gene expression during the biofilm mode of growth

Genetic and molecular studies of bacterial communities have helped us to get a better understanding of the various stages of bacterial attachment and biofilm development, mechanisms of antimicrobial resistance and in formulating methods to control biofilm infections. The first report of gene expression owing to a biofilm phenotype was made using *lacZ* reporter-gene fusions (Davies *et al.,* 1993). A phenotypic shift in behavior in conjunction with differential regulation of a large array of genes is associated with cells when they switch from planktonic to biofilm mode of growth (Whiteley *et al.,* 2001; Prigent-Combaret *et al.,* 1999). In spite of the distinguishable characteristics of these two modes of growth, only 1% of genes of *P. aeruginosa* showed differential expression between them, whereby 0.5% of genes were expressed and 0.5% were repressed in the sessile mode (Whiteley, *et al.,* 2001). However, based on a review of several studies Sauer (2003) reported that the up- and down-regulation of genes could vary between 1% and 38% of the total genome and may be just a transient snapshot of gene

expression which may not necessarily be correlated to the phenotype. It has also been demonstrated that various proteins are involved in different stages of biofilm development. Surface-associated proteins, such as SpaP and Fap1 (Bowen *et al.,* 1991; Froeliger and Taylor 2001) and glucan binding proteins (Sato *et al.,* 1997) such as GbpA, GbpB, and GbpC play an important role in the initiation and formation of biofilms by oral streptococci. Bacterial cell surface proteins or extracytoplasmic proteins such as ClpP play a role in the initial attachment of bacteria to abiotic surfaces and biofilm formation (O'Toole and Kotler, 1998). Structural proteins involved in adhesion and autoaggregation, such as OmpC, OmpF, OmpT, *lpxC* (encoding a protein associated with lipid A biosynthesis), and *slp* (encoding an outer-membrane lipoprotein) were over-expressed in the biofilm growth compared with planktonic growth (Schembri *et al.,* 2003), although no genes regulated in response to quorum sensing were found. A global proteome analysis of *P. putida* cells after a 6 h attachment period to a silicone surface was carried out using 2-D protein gel electrophoresis and cDNA subtractive hybridization techniques (Sauer and Camper, 2001). Their study revealed 45 differences in the protein profiles of planktonic and sessile cells, wherein those involved in amino acid metabolism, OM lipoprotein, putative ABC transporter (PotF) and proteins involved in flagellar synthesis were down-regulated, whereas proteins for synthesis of type IV pili and lipopolysaccharides and the ABC transporter (PotB) were up-regulated in the attachment mode. Through a DNA microarray study, Whiteley *et al*. (2001) demonstrated that appendages like pili and flagella were not required during the maturation and maintenance stages of biofilm development; whereas, the TolA membrane protein and OmlA membrane lipoprotein were induced in the biofilm cells. RpoS, the σ^S subunit of RNA polymerase, governs the expression of many genes during the stationary phase, and deletion of *rpoS* rendered *E. coli* incapable of establishing sessile communities (Schembri *et al.,* 2003). However, an opposite effect was seen in *P. aeruginosa* wherein the *rpoS* gene was repressed in biofilms and rpoS-deficient mutants formed thicker biofilms than the wild type cells and were more resistant to antimicrobial treatment (Whiteley *et al.,* 2001).

Using *in vivo* expression technology, five new essential genes were identified for *P. aeruginosa* biofilms (Finelli *et al.,* 2003) which had not been detected in previous DNA microarray analyses. Using GeneChip Expression analysis, several SOS stress response genes were found to be expressed in *E.coli* persister cells (*recA,*

sulA, uvrBA, and umuDC); the phage-shock (psp) operon genes; and several heat and cold shock genes (*cspH, htrA, ibpAB, htpX*, and *clpB*) and those belonging to TA modules *dinJ/yafQ, yefM, relBE*, and *mazEF* (Keren *et al.,* 2004). Hefford *et al*. (2005) used a 2-dimensional polyacrylamide gel electrophoresis technique to study the proteomes of biofilm- and planktonically-grown *Listeria monocytogenes* and found that the proteins involved in stress response, envelope and protein synthesis, biosynthesis, energy generation, and regulatory functions were highly-expressed in biofilm-grown cells. Very recently, Mosier *et al*. (2014) used tandem mass tag (TMT)-based proteomics to investigate the proteomic response of individual organisms within a biofilm community to elevated temperatures, thus paving the way for studies of gene expression in mixed culture and environmental samples.

2.8. Gene expression during antimicrobial treatment of microbial biofilms

A review of published literature shows that several genes can be expressed upon antimicrobial treatment and will depend on several factors such as the type of antimicrobial and microorganism, treatment conditions, concentration of the antimicrobial and exposure time. Exposure of *P. aeruginosa* biofilms to high concentrations of tobramycin resulted in expression of 20 genes that may be responsible for biofilm resistance to this antibiotic (Whiteley *et al.,* 2001). DNA microarray technology was used in this study to gain possible insights into bacterial antibiotic resistance mechanisms. Among them, the role of *tolA, rpoS* and cytochrome *c* oxidase in antibiotic resistance was discussed in detail. Other proteins induced were hypothetical proteins of unknown function, efflux proteins and stress response proteins (dnaK and groES). Activation of genes encoding efflux pumps was suggested to be responsible for *P. aeruginosa* biofilm resistance to antibiotics (Greenberg, 2000; Whiteley *et al.,* 2001). Zhang *et al*. (2013) characterised three specific antibiotic resistance loci in *P. aeruginosa* biofilms. They included *ndv*B that encodes a glucosyltransferase, important for the synthesis of cyclic-β 1, 3 glucans, an operon that encodes an efflux pump and tssC1 through an unknown mechanism.

Proteomic studies on *Delftia* biofilms and on biofilms treated with CHX are very limited. The first study

on global transcriptomic response of *P. aeruginosa* treated with CHX was published in 2009 by Nde and co-researchers (Nde *et al.,* 2009). They studied the response of *P. aeruginosa* to treatment with 0.008 mM CHX diacetate for 10 and 60 min using *P. aeruginosa* GeneChip arrays and found that out of a total of 5900 genes, 250 genes were statistically-significantly up-regulated (≥2-fold) or down-regulated (≤2-fold). A few studies have indicated that the most prominently down-regulated genes under CHX stress were those encoding heat shock proteins (Nde *et al.,* 2009; Hassana *et al.,* 2013). Apart from these, the other genes that were down-regulated in the study of Nde *et al*. (2009) at both exposure times (10 and 60 min) to CHX were membrane transport, oxidative phosphorylation and electron transport genes. The *mex*C and *mex*D genes of the MexCD-OprJ multidrug efflux pump were significantly up-regulated after both treatment times. On the other hand, only 22 and 35 genes were up- and down-regulated by more than two-fold, respectively, when *Acinetobacter baumannii* cells were exposed to 4 mg L^{-1} CHX in a whole genome microarray study (Hassana *et al.,* 2013). In yet another microarray study (Coenye *et al.,* 2011), of the 7,153 protein-coding *B. cenocepacia* J2315 genes, 469 (6.56%) were significantly over-expressed by more than two-fold in CHX-treated sessile cells, while 257 protein-coding genes (3.59%) were significantly down-regulated. The majority of over-expressed genes encoded periplasmic and lipoproteins and were required for the structure and function of the inner membrane, were involved in transport or binding, had regulatory functions, or were involved in chemotaxis and motility. The majority of down-regulated genes in sessile CHX-treated *B. cenocepacia* J2315 cells were involved in transport or binding or had a regulatory function similar to the findings of Nde *et al*. (2009). In a comparative study of the action of CHX on membrane proteins of Gram positive (*Bacillus subtilis*) and Gram negative bacteria (*E. coli*), Cheung *et al*. (2012) found that five proteins related to purine nucleoside interconversion and metabolism were preferentially expressed in the cell wall of *E. coli*, while three proteins related to stress response and four others in amino acid biosynthesis were up-regulated in the cell wall of *B. subtilis*. In a very recent comparative analysis of CHX-sensitive and CHX-tolerant strains of *Salmonella typhimurium*, Condell *et al*. (2014) reported the involvement of a CHX defense network in both cases. The defense network involved multiple cell targets such as those associated with the synthesis and modification of the cell wall, the SOS response, virulence, and an anoxic pathway metabolism with no involvement of efflux

systems. In addition to extensive modifications of these cellular processes, up-regulation of the flagellar apparatus and altered phosphate metabolism was seen in CHX-tolerant cells. In the same study, using the differential in gel electrophoresis (DIGE) technique, the authors reported that after a sub-lethal exposure to CHX, a total of 247 proteins were significantly differentially-expressed (>2-fold-change, $p < 0.05$); 81 of these genes were up-regulated whilst 166 were down-regulated in the CHX-sensitive strain. Some of the proteins associated with cellular permeability that were expressed in CHX-treated *Salmonella* wild type cells included those involved in the biosynthesis and cross-linking of the peptidoglycan subunits (*murG*, *murD*), thus affecting the efficacy of CHX action and/or transport. The O-antigens that provide anionic binding sites were down-regulated, thus altering CHX binding to the cells. Up-regulation of the DNA repair system was also observed in keeping with other studies (Nde *et al.,* 2009) to mitigate the DNA cross-linking effects of CHX with a decrease in protein synthesis.

2.9. The Tol System

The extracellular toxins produced by *E. coli* and *Pseudomonas* species (i.e., colicins, pyocins) must cross the membrane barrier of the target cells to exert their lethal action. In order to achieve this, they must first recognize a specific receptor in the bacterial OM and then translocate through the cell envelope. Colicins, and similarly, single-stranded DNA of some filamentous bacteriophages use two different translocation systems to cross the cell envelope; namely, the Ton and Tol systems. Dennis *et al*. (1996) reported the first *Pseudomonas tol* mutant that was tolerant to pyocin; however, most of the studies in this area have taken place in the last decade. Published data on the Tol system is scarce and is mostly based on *E. coli* and *Pseudomonas* with very limited studies on other species, namely *Vibrio* (Heilpern and Waldor, 2000; *Salmonella* (Prouty *et al.,* 2002) and *Haemophilus* (Sen *et al.,* 1996). The Tol system is commonly referred as Tol-Pal and Tol-OprL in *E. coli* and *Pseudomonas*, respectively. They are composed of seven proteins that are associated with different cellular compartments as shown below (Table 2.1):

Table 2.1 Tol system in *E. coli* and *Pseudomonas* sp.

Location	**In *E. coli***	**In *Pseudomonas sp*.**
Outer membrane	Pal	OprL
Periplasm	TolB and YbgF	Tol B and Orf2
Inner membrane	TolQ, TolR, TolA	TolQ, TolR, TolA
Cytoplasm	YbgC	Orf1

Sturgis (2001) investigated the distribution of the *tol-pal* gene cluster and found them in the genomes of 10 different Gram negative bacteria. The gene cluster was not found in any Archaea, spirochete or Gram positive bacteria. Absence of the cluster in the Gram positive bacterial genomes implies their role in OM integrity and their absence in some Gram negative genomes indicates that the cluster is not universal. Interestingly, the structure of the *tol-pal* operon was similar in different species, wherein tolQ, tolR and tolA genes were easily identifiable (Fig 2.2).

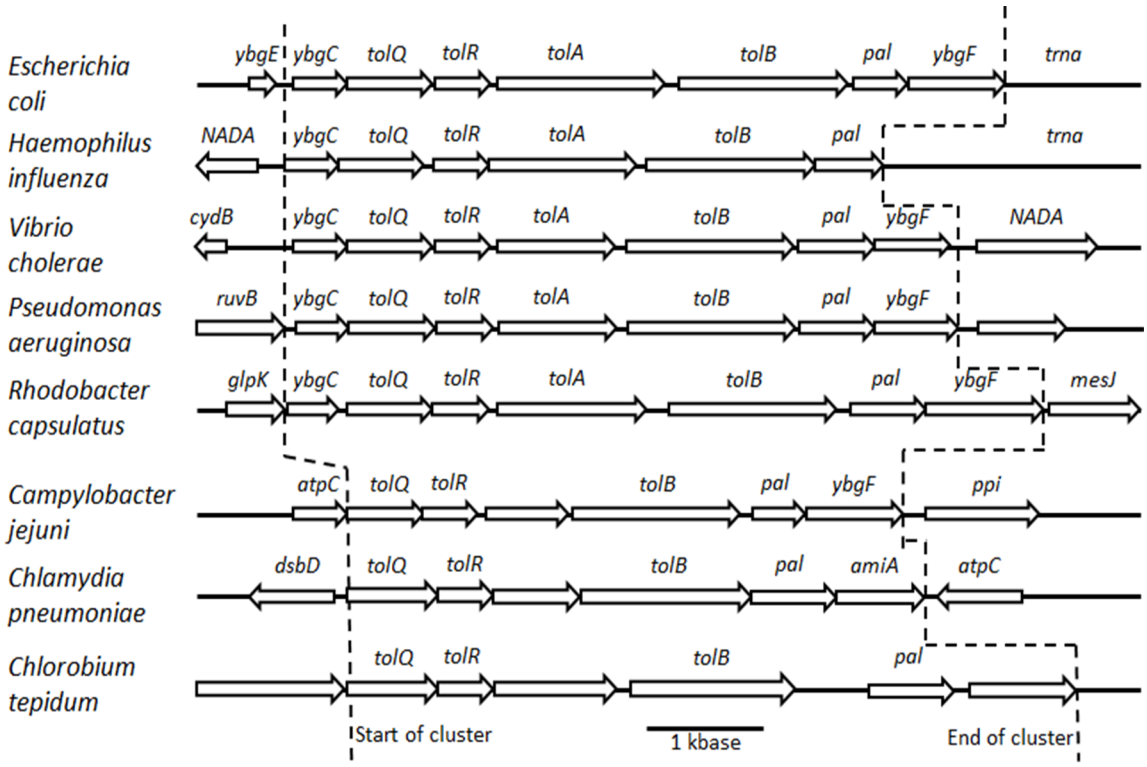

Figure 2.2 Organisation of the *tol-pal* genes cluster in different species (adapted from Sturgis, 2001).

In spite of their existence in several Gram negative bacteria, the transcriptional organisation of these genes varies between different species, and in fact, it is seen to vary even within the same genus. Furthermore, in their review, Rodriguez-Herva and Llamas (2004) concluded that the failed attempts to determine the transcriptional initiation points of the *tol-oprL* (*pal*) promoters in *E. coli* and *P. aeruginosa* could be due to the low level of transcription and the instability or rapid degradation of the transcribed products. In *E. coli,* this system is organised into two operons, with YbgC, TolQ, TolR, TolA, TolB, Pal and YbgF forming the first operon and the second one from an inner promoter encoding TolB, Pal and YbgF (Fig. 2.2) (Vianney *et al.,* 1996; Muller and Webster, 1997). On the other hand, in *P. putida* the *tol-oprL* genes are arranged in two transcriptional units, *orf1-tolQ-tolR-tolA-tolB* and *oprL-orf2* and in *P. aeruginosa* 3 units may occur; *orf1-tolQ-tolR-tolA*; *tolB* and *oprL-orf2* (Rodriguez-Herva and Llamas, 2004). The stoichiometry of the Tol-Pal complex is poorly-defined, although it is known that each gene is expressed at different levels. TolA and TolR abundance have been measured as about 600 (Levengood *et al.,* 1991) and 2500 (Muller *et al.,* 1993) copies per cell, respectively, and Pal is the most abundant with an

estimate of 8000-40000 copies per cell (Sturgis *et al.,* 2001). Guihard *et al*. (1994) estimated a ratio of 2.7: 1 between TolQ to TolR. In *P. aeruginosa*, the number of amino acids and the molecular weight of TolQ, TolR and TolA are 231, 146, 346 and 25, 250; 15, 788; 37, 813 daltons, respectively, and sequence identity to *E. coli* TolQ, TolR and TolA were 53, 38 and 27%, respectively (Dennis *et al.,* 1996). The expression of these *tol-pal* genes is tightly-regulated. For example, *tolR* is only expressed following the successful translation of *tolQ*, allowing the production of these proteins to be co-ordinated, which is relevant as they form a complex together (Muller *et al*. 1993).

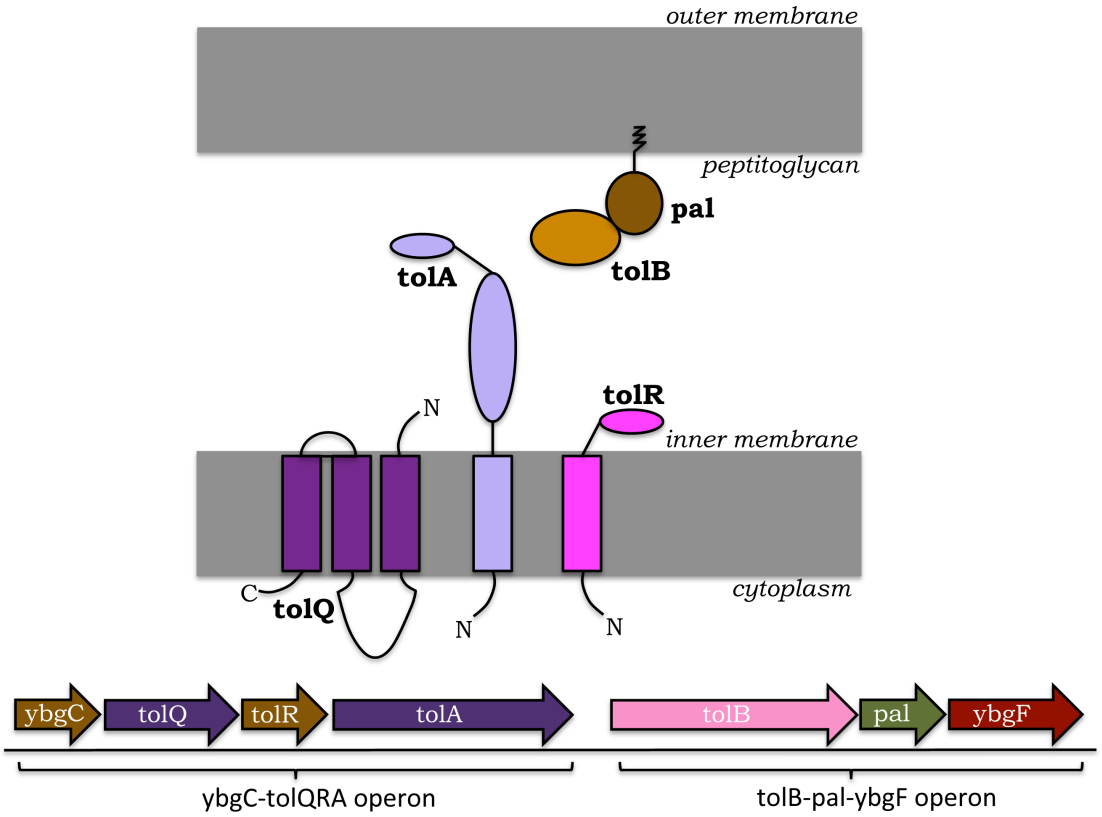

Figure 2.3 Schematic representation of the various genes in the Tol system of *E. coli* showing their locations and transmembrane helices and the two operon structure (http://www.proteopedia.org/wiki/images/3/31/TOL.jpg)

2.9.1. TolQ

In *E. coli*, TolQ is an integral membrane protein of 25.5 kDa containing 230 amino acids with three

transmembrane helices and a large cytoplasmic domain between helices 1 and 2 (Fig. 2.3) (Sun and Webster, 1987). Interactions within the cytoplasmic membrane have been found between TolQ and the other two integral membrane proteins, TolA and TolR. TolQ is mainly localised in the inner membrane and trace amounts can be present in the adhesion sites between the inner and outer membrane (Bourdineaud *et al.,* 1989). The study by this group also revealed the direct involvement of the *tolQ* gene product in the entry of colicin A and that only a small amount of the TolQ protein was required for this purpose, wherein any excess protein was rapidly degraded. It has been demonstrated that the transmembrane domains of TolQ and TolR have structural and functional homologies not only to ExbB and ExbD of the TonB system but is also extended to MotA and MotB of the flagellar motor (Cascales *et al.,* 2001).

The *P. aeruginosa* TolQ has 53% homology to *E. coli* TolQ (Dennis *et al.,* 1996) and is one of the most conserved proteins in Pseudomonads and other Gram negative bacteria. In this study, transcriptional analysis of the *tol* genes revealed that the *tolQ* and *tolR* genes are co-transcribed as an approximately 1.5-kb transcript in *P. aeruginosa* and that tolA is transcribed from its own promoter as an approximately 1.2-kb gene product. They also demonstrated that *P. aeruginosa* Tol proteins were functionally unable to complement *E. coli tol* mutants. In an earlier study, it was shown that the *tolQ* mutation had a strong polar effect on the expression of *tolR* (Vianney *et al.,* 1996) and that TolR stability required the presence of TolQ (Cascales *et al.,* 2001) and further, both TolQ and TolR proteins are essential for the TolA-Pal interaction. OM defects were noticed in *tolQ* mutants in terms of increased OM vesicle formation, RNaseI leakage and sensitivity to SDS and vancomycin (Cascales *et al.,* 2001).

2.9.2. Function of the Tol system

The precise function of Tol proteins is unclear; however, they appear to be involved in maintaining the integrity of the cell envelope. Though it is known that the Tol import system plays some role in the translocation of certain biological macromolecules across the cellular membrane, the molecular interactions between these components is still unclear. Experiments have also shown that these proteins are required for single-stranded DNA

filamentous bacteriophages to translocate DNA into the cytoplasm (Sun and Webster, 1987). Tol mutations have resulted in phenotypes hypersensitive to certain drugs and detergents, resistant to colicins, releasing periplasmic proteins (Lazdunski *et al.,* 1998), and producing small OM vesicles (Bernadac *et al.,* 1998). Studies also suggest that the Tol/Pal system might anchor the OM to the peptidoglycan layer (Clavel *et al.,* 1998) and might catalyse porin biogenesis or regulate porin activity (Dover *et al.,* 2000). Mutation in the *tolA* gene caused changes in the LPS of *P. aeruginosa* cells making them more susceptible to aminoglycosides (Rivera *et al.,* 1988) by increasing their affinity to the OM. The *tolA* gene was activated when *P. aeruginosa* biofilms were exposed to aminoglycosides, indicating their possible role in antibiotic resistance (Whiteley *et al.,* 2001). In addition, *tol*-minus *E. coli* cells could not separate into mother and daughter cells during cell division and formed long chains of cells (Bernadac *et al*. 1998) (Cascales *et al.,* 2001) (Gerding *et al.,* 2007). As Tol proteins may form some kind of trans-envelope bridge from outer to inner membrane, it has been proposed that Tol proteins may act to pull the outer and inner membranes together during cell division, as part of the invagination process (Gerding *et al*. 2007). In a recent study, Teleha *et al*. (2013) demonstrated the involvement of Tol proteins in cell division and over-expression of TolQ resulted in an altered phenotype in E. coli cells (elongated and in long chains). The role of Tol-Pal (OprL) system in the transport of various carbon sources across the cytoplasmic membrane has also been described in *Pseudomonas* and E. coli (Llamas *et al.,* 2003).

In conclusion, further insights in CHX tolerance in *D. acidovorans* can be examined using a combination of microscopic and molecular studies.

3. MICROSCOPIC AND SPECTROSCOPIC ANALYSES OF CHLORHEXIDINE-TOLERANCE IN *DELFTIA ACIDOVORANS* BIOFILMS.

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Author contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. Preparation of the initial draft of the manuscript, as well as all other data presented in this manuscript, are the work of the thesis author.

3.1. Abstract

The physicochemical responses of *Delftia acidovorans* biofilms exposed to the commonly-used antimicrobial, chlorhexidine (CHX), were examined. A CHX-sensitive mutant (Minimum Inhibitory Concentration; MIC = 1.0 μg ml^{-1}) was derived from a CHX-tolerant (MIC = 15.0 μg ml^{-1}) *D. acidovorans* parent strain using transposon mutagenesis. Mutant (MT51) and wild-type (WT15) *D. acidovorans* biofilms were cultivated in flow cells and then treated with CHX at sub-MIC and inhibitory concentrations and examined by confocal laser scanning microscopy (CLSM), scanning transmission x-ray microscopy (STXM) and infrared (IR) spectroscopy. Specific morphological, structural and chemical compositional differences between the CHX-treated and untreated biofilms of both strains were observed. Apart from architectural differences, CLSM revealed a negative effect of CHX on biofilm thickness in the CHX-sensitive MT51 biofilms relative to the WT15 strain. STXM analyses showed that WT15 biofilms contained two morpho-chemical cell variants; whereas, only one type was detected in MT51 biofilms. Cells in MT51 biofilms bioaccumulated CHX to a similar extent as one of the cell types found in WT15 biofilms; whereas, the other cell type in WT15 biofilms did not bioaccumulate CHX. STXM and IR spectral analyses revealed that CHX-sensitive MT51 cells accumulated the highest levels of CHX. Pre-treatment of biofilms with EDTA promoted accumulation of CHX in all cells. Thus, it is suggested that a sub-population of cells that do not accumulate CHX appear responsible for greater CHX resistance in WT15 *D. acidovorans* biofilm in conjunction with the possible involvement of bacterial membrane stability.

3.2. Introduction

Antimicrobials are extensively used in clinical, domestic and industrial applications resulting in their continuous release into the environment, usually via sewage effluents. The presence of these compounds in the environment has led to concerns regarding selection of bacterial strains that have reduced susceptibility towards various kinds of antimicrobials (Fraise, 2002). Chlorhexidine (CHX) is a widely-used broad-spectrum

antimicrobial agent and its implications for microorganisms and the environment have received varying degrees of attention since its introduction (Lawrence *et al.,* 2008). Chemically a cationic, hydrophobic and lipophilic bisbiguanide ($C_{22}H_{30}Cl_2N_{10}2C_6H_{12}O_7$), CHX is an active ingredient in many 'daily-use' disinfectants, antiseptics and pharmaceutical preservatives. CHX possesses broad antibacterial and antifungal activity. It reacts with negatively-charged phosphate groups on microbial cell walls causing membrane damage, enzyme inhibition and leakage of cytoplasmic constituents (Hope and Wilson, 2004). Extensive cell damage, coagulation of cytoplasmic constituents, and precipitation of proteins and nucleic acids can be expected when high concentrations (2%) of CHX are used due to the interactions between CHX and phosphorylated cytoplasmic molecules (Hope and Wilson, 2004; Mohammadi and Abbott, 2009).

Although several microbes have been reported to survive in the presence of in-use concentrations of CHX (Brooks *et al.,* 2002, Marrie and Costerton, 1981, Stickler *et al.,* 1987), little is known about the underlying mechanisms for this tolerance. Resistance to biocides is often referred to as tolerance (Favero, 2002). Biocides usually have multiple cellular targets and their effects are concentration dependent and often evaluated in terms of their ability to reduce the numbers of viable microorganisms. An earlier study indicated that CHX inhibits Gram-positive and Gram-negative bacteria at concentrations of 1 mg L^{-1} and 2-2.5 mg L^{-1}, respectively (Russell and Day, 1993). However, MIC values for CHX vary depending on the type of bacteria, phenotypic and genotypic status of the organism and the environmental conditions. Tolerance levels could be much higher when microbes grow as biofilms (Hoiby *et al.,* 2010; Mah and Toole, 2001). Biofilms are assemblages of microorganisms that form at interfaces, surrounded by self-produced extracellular polymeric matrices, typically attached to abiotic or biotic surfaces (Hoiby *et al.,* 2010). General mechanisms of antimicrobial resistance in biofilm bacteria have been described and discussed in scientific journals (Hoiby *et al.,* 2010; Mah and Toole, 2001; Lewis, 2001). However, this literature largely consists of phenomenological descriptions of CHX resistance in biofilm communities rather than examining the specific mechanisms involved. Some bacterial genera (i.e., *Proteus*, *Providencia*) may either be intrinsically-resistant to CHX or have induced mechanisms of resistance due to phenotypic and genotypic changes (Russell, 1995). Penetration failure within biofilms, permeability characteristics of the cellular outer membrane

(Gilbert and Moore, 2005), CHX degradation (Tanaka *et al.,* 2005) and the presence of resistance genes or plasmids have all been reported as possible causes for CHX resistance.

Developments in microscopy have enabled researchers to develop a better understanding of biofilms and to study their interactions with various antimicrobials (Mangalappalli-Illathu and Korber, 2006; Neu *et al.,* 2010; Vitkov *et al.,* 2005). Microscopic techniques such as confocal laser scanning microscopy (CLSM), transmission electron microscopy (TEM) and scanning transmission X-ray microscopy (STXM) (Hitchcock, 2012) have been used singly, or in combination, to examine complex microbial communities and to map the extent, nature and distribution of macromolecules in a biofilm (Dynes *et al.,* 2009; Lawrence *et al.,* 2003). Cell viability, cell morphology, biofilm architecture and matrix composition can be studied in living, fully hydrated systems using CLSM (Lawrence *et al.,* 2003; Korber *et al.,* 1994). Fluorescent dyes such as SYTO9 and propidium iodide can be employed in combination with CLSM to evaluate the potential for antimicrobial agents to influence membrane integrity and permeability (20). The distribution and concentration of CHX in combination with biomacromolecules (e.g., proteins, lipids, polysaccharides) can be investigated using STXM at a spatial resolution of <30 nm in hydrated biofilm systems (Dynes *et al.,* 2009; Dynes *et al.,* 2006). Sequences of images recorded over a span of photon energies at core excitation edges can be converted to quantitative maps of these compounds using suitable quantitative reference spectra. STXM at the C 1s edge has been exploited in several studies for mapping major biomacromolecules in biofilms (Dynes *et al.,* 2009; Lawrence *et al.,* 2003; Lawrence *et al.,* 2012; Liu *et al.,* 2013). In addition, vibrational signatures of chemical bonds in biomolecules within a biofilm with a spatial resolution of <10 μm can be obtained using synchrotron radiation-based Fourier transformed infrared spectroscopy (SR-FTIR). Most of the relevant biological information can be obtained from the mid-infrared region (4000-800 cm^{-1}). Infrared absorption spectra of organic molecules provide unique "fingerprints" that can be applied for identification purposes with great reliability (Suci *et al.,* 1998). Bacterial biofilm changes that occur during: i) adaptation to their surrounding environments, ii) transportation, adsorption and interaction kinetics of antimicrobial agents within the biofilms (Holman *et al.,* 2009; Susi *et al.,* 2001; Suci *et al.,* 1994), and iii) biofilm developmental stages (Quiles *et al.,* 2010) have also been monitored using SR-FTIR.

Delftia acidovorans, formerly known as *Comamonas acidovorans* and *Pseudomonas acidovorans*, is a Gram-negative bacillus ubiquitously found in soil and water. Previously considered to be non-pathogenic, reports of *D. acidovorans* association with a number of serious infections, including bacteremia, empyema, bacterial endocarditis, ocular and urinary tract infections (Cho and Lee, 2002; del Mar Ojeda-Vargas *et al.,* 1999; Horowitz *et al.,* 1990; Perla and Kuntson, 2005), have been increasing, thus establishing this organism as an emerging opportunistic pathogen. Studies on the effects of antimicrobial agents on *D. acidovorans* biofilms are very limited and the available CHX literature has focused on either clinical isolates or dental biofilms. Thus, the effects on environmental *D. acidovorans* biofilms have never been reported.

In the present study, a combination of CLSM, STXM and SR-FTIR spectroscopy was applied to evaluate the effects of sub-MIC and inhibitory levels of CHX on CHX-tolerant and -sensitive *D. acidovorans* biofilms to elucidate intrinsic mechanisms of CHX resistance that may exist.

3.3. Materials and methods

3.3.1. Isolation, identification, mutant development and MIC determination

The wild-type *Delftia acidovorans* (WT15) strain used in this study was isolated from South Saskatchewan River water biofilms during an earlier study on antimicrobial resistance (Mooney, 2006). The isolate was identified by a 16S rRNA gene sequencing protocol described elsewhere (Hirkala and Germida, 2004). Sequencing was carried out at the Plant Biotechnology Institute (PBI), National Research Council of Canada, Saskatoon, SK. Sequence identity was confirmed using Basic Local Alignments Search Tool (BLAST) and Ribosomal Database Project's Sequence Match (RDP) comparing the obtained sequence to species with a greater than ≥ 99 % match. Mutants were created from the WT15 strain using the EZ-Tn5^{TM} <KAN2> Tnp Transposome kit (Epicentre Biotechnologies, Madison, WI) following the supplier's instructions and screened for sensitivity to CHX.

The gene interrupted by transposon (Tn5) insertion in *D. acidovorans* (MT51) was identified by the rapid amplification of transposon ends (RATE) technique, as described by Karlyshev *et al.* (2000). Amplified products were analysed by electrophoresis on a 1% agarose gel in 1X TAE buffer containing 0.5 μg ml^{-1} ethidium bromide. The product band was purified using the QIAquick Gel Purification kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions and then sent to the National Research Council of Canada, Saskatoon, Saskatchewan, Canada for sequencing. The gene disrupted by Tn5 insertion was identified as *TolQ*, a component of the *TolQRAB* gene cluster known to be involved in outer membrane stability in many bacterial species. The sequence of the disrupted gene (*tolQ*) from WT15 was then compared with sequences published in the NCBI Nucleotide Database. The sequence data for *TolQ* was submitted to the GenBank database under accession number KT988307. TolQ has been shown to be an integral cytoplasmic membrane protein required for maintenance of the integrity of the bacterial envelope in *E. coli* (Vianney *et al.,* 1994).

The minimum inhibitory concentration (MIC), defined as the lowest concentration of CHX sufficient to inhibit the growth of the bacteria, was determined for the WT15 and selected mutants by the micro-dilution method (Andrews, 2001) using different concentrations of CHX dihydrochloride (Sigma Aldrich Co., St. Louis, MO). The mutant (MT51) selected for this study had a CHX MIC = 1.0 μg ml^{-1}, compared to a 15 μg ml^{-1} MIC for the WT15 parent strain.

3.3.2. Bacteria and culture conditions

Delftia acidovorans (WT15 and MT51) strains were grown from frozen stock cultures stored in 10% (vol/vol) glycerol at -80°C (Kirsop and Snell, 1984) on Tryptic Soy Agar (TSA) plates and incubated overnight at room temperature (RT; 23±2° C). A well-isolated colony was transferred to 50 ml of 1% of full strength (30 g L^{-1} Tryptic Soy Broth (TSB) in an Erlenmeyer flask and incubated on a gyratory shaker (150±5 rpm) at RT until the mid-log phase of growth was reached (at approximately 14 h). This log phase culture was then used to inoculate the flow cells set up for the study.

3.3.3. Cultivation of biofilms for confocal laser scanning microscopy (CLSM) analysis

Multichannel flow cells for CLSM biofilm analysis were constructed as previously described (Korber *et al.,* 1994). The flow cell apparatus was sterilized with 5.25% (wt/vol) sodium hypochlorite solution for at least 15 min and then flushed using sterile water followed by sterile growth medium (1% of full strength TSB or 100 times-diluted normal TSB). Biofilms of WT15 and MT51 strains were cultivated by injecting 0.5 ml of log phase cells, prepared as outlined above, into each channel of the flow cell. During inoculation, the flow cells were left undisturbed for 30 min to facilitate attachment of the cells to the flow cell surface, after which pumping of the growth medium was resumed thereby washing out unattached cells. Growth medium (1% TSB) was continuously pumped through each flow cell channel at a bulk flow rate of 25 ml h^{-1} (0.07 cm sec^{-1} laminar flow velocity) using a peristaltic pump (Watson Marlow 202U, Cornwall, UK) for 24 h. To evaluate the effect of CHX, established (24 h) biofilms were then continuously treated for another 24 h period with sterile 1% TSB supplemented with either 0 (Control), 10 μg ml^{-1} (sub-lethal) or 30 μg ml^{-1} (inhibitory) CHX.

3.3.4. Cultivation of biofilms for STXM analysis

Flow cells for STXM analysis were constructed as previously described (Dynes *et al.,* 2006). Briefly, a silicon nitride window (Silson Ltd., Blisworth, UK) was placed between two glass microscope slides modified to accommodate inlet and outlet tubing, sealed with silicon adhesive and then the entire flow cell apparatus was sterilized by autoclaving.

Inoculation and growth conditions (i.e., medium, pumping) were as outlined above. The biofilm cultivation period for STXM analysis was 12 h instead of the 24 h used in the CLSM study, as STXM analysis is limited to a maximum biofilm thickness of <10 μm. The 12 h biofilms were treated for a further 12 h with sterilized medium supplemented with either 0 or 10 μg ml^{-1} CHX, after which the windows were removed from the flow cells, air-

dried and fixed to the STXM sample holder. In a separate experiment, to examine the effect of inhibitory CHX concentrations, the 12 h (cultivation period) biofilms were treated with sterilized medium supplemented with 30 μg ml^{-1} and 100 μg ml^{-1} of CHX for 0.5 and 1 h. The windows were then removed from the flow cells, air-dried and fixed to the STXM sample holder. To further study CHX accumulation over time, the 12 h biofilms on silicon nitride windows were treated with sterilized medium supplemented with 30 μg ml^{-1} of CHX and removed from the flow cells after 1 and 12 h of CHX exposure, air-dried and fixed to the STXM sample holder.

Lastly, to investigate the effect of CHX on cell membrane-permeabilised biofilms, WT15 biofilms were grown for 12 h using sterilized medium supplemented with 1 mM ethylenediaminetetraacetate (EDTA), a known membrane permeabiliser, and then continuously treated for 12 h with sterilized medium supplemented with 10 μg ml^{-1} CHX, after which the windows were removed, air-dried and fixed to the STXM sample holder. Untreated biofilms (no CHX) were compared as control.

3.3.5. Cultivation of biofilms for IR spectroscopy

The flow cell setup for IR analysis was similar to that used for STXM analysis except that the biofilms were grown directly on IR slides (Kevley Technologies, Chesterland, Ohio) with the internal reflection element as the substratum. Accordingly, the IR slide was sealed with silicone adhesive to a microscope glass slide having inlet and outlet ports. The flow cells were inoculated as described earlier, incubated under flowing conditions for 24 h and then treated with sterilized medium supplemented with either 0 (control), 10 or 30 μg ml^{-1} CHX, for an additional 24 h. The IR slides with attached biofilms were then removed and air-dried prior to IR analysis.

3.3.6. Confocal laser scanning microscopy and data analysis

CLSM was used in conjunction with the *Bac*Light LIVE/DEAD staining kit (Molecular Probes, Life

Technologies, Burlington, ON) i.e., Syto9 (live) and propidium iodide (dead) to quantify *D. acidovorans* viability in biofilms following 24 h CHX treatment, as follows. Flow cells were mounted on the stage of the confocal microscope and treated with of the LIVE/DEAD stain for 5 min in accordance with the manufacturer's instructions. Optical thin sections were collected at 1 μm intervals from the attachment surface at five randomly-chosen locations in each flow cell channel using a Bio-Rad MRC-1024 Lasersharp fluorescence scanning confocal laser system (Carl Zeiss MicroImaging GmbH, Jena, Germany). Dual-channel images, corresponding to fluorescence emission in the green (excitation/emission 488/522) (Syto9) and red (excitation/emission 535/617) (propidium iodide) wavelengths, were acquired in either the *xy* (as indicated above) or vertical *xz* planes. Digital image analysis (NIH image software, version 1.63f; National Institutes of Health, Bethesda, MD) of the optical thin sections was then used to determine cell viability and live/dead ratios (% fluorescence) at 48 h for both control and CHX-treated biofilms. Biofilm thickness was measured in micrometers with the aid of a computer-controlled, motorized *z*-axis stepper motor and manual focusing with a Nikon Microphot-FXA microscope (Nikon Corp., Tokyo, Japan) (Neu *et al.,* 2010). Average thickness values were calculated from 5 random fields for each biofilm, with thickness values measured at 5 separate locations per field. This analysis was experimentally replicated in triplicate for a total of 75 thickness measurements per treatment.

3.3.7. Scanning transmission x-ray microscopy (STXM) and data analysis

X-ray imaging and spectromicroscopy of biofilms prepared as described above were carried out using the STXM microscopes on beamlines 5.3.2.2 at the Advanced Light Source (ALS) (Berkeley, CA) (Kilcoyne *et al.,* 2003) and the SM beamline 10ID-1 at the Canadian Light Source (CLS) (Saskatoon, SK) (Kaznatcheev *et al.,* 2007). Near edge X-ray fine structure absorption spectra (NEXAFS) were collected at the C 1s edge at a single energy, or as a sequence of energies (i.e., an 80 to 100 image stack) from 280 to 320 eV. The raw transmitted signals were converted to optical densities (absorbances) using the incident flux signals measured at regions without biofilm, correcting for absorbance by the silicon nitride windows. Quantitative component maps of the

major biomacromolecules (protein, lipid, polysaccharide) as well as CO_3^{2-}, K^+ and CHX, were derived by fitting the image sequences at each pixel to the spectra of reference compounds that had been placed on an absolute linear absorbance scale using the singular value decomposition procedure (Kirsop and Snell, 1984). Absolute linear absorbance is the optical density (OD) per unit path length of a pure material of defined density, where the absorbance (A), also called OD, is given by A= OD = -ln(I/Io), where I = transmitted intensity and Io = incident intensity, and is established by adjusting the intensity scale of the reference spectrum to that of the computed elemental response outside the structured near edge region (Dynes *et al.,* 2006). The lower and upper limits of the gray scale indicated in the component maps are a measure of the component thickness in nm. The reliability and methodology used to quantitatively map the major biomacromolecules in biofilm cells has been described in detail elsewhere (Hitchcock, 2012; Dynes *et al.,* 2006; Dynes *et al.,* 2006). The reference compounds used were protein (human serum albumin), lipid (1, 2-dipalmitoyl-*sn*-glycero-3-phosphocholine), polysaccharide (xanthan gum), carbonate (calcite), K (K_2CO_3 with CO_3 derived from Ca carbonate subtracted) (Korber *et al.,* 1994; Dynes *et al.,* 2009) and chlorhexidine dihydrochloride (CHX). The microscope energy scale was calibrated to ± 0.05 eV accuracy using the Rydberg peaks of CO_2. The analysis was carried out using Axis2000 software (Hitchcock, 2014).

3.3.8. Infrared spectroscopy and data analysis

Air-dried 48 h CHX-treated and control biofilms were analyzed using the Mid Infrared Spectromicroscopy (Mid-IR) Beamline 01B1-1 at the Canadian Light Source, Saskatoon, using a Bruker Optics IFS66v/s FTIR spectrometer and a Hyperion 2000 IR microscope. The slides were mounted on the microscope and the area to be analysed was focused, after which the chamber was tightly-closed and flushed with nitrogen gas for at least 15 min to minimize CO_2 and water vapour interference. The instrument was operated using the following settings: reflectance mode, phase resolution of 32 cm^{-1}, scanner velocity of 8, 40.0 KHz and an aperture size of 12 µm x 12 µm (±3 µm). The samples were scanned over a wide spectral range i.e., from 4000 cm^{-1} to 800 cm^{-1}. Single point

background scans (n=5000) were taken on the IR slide that was free of biofilm material to establish the background noise level. The biofilm samples were scanned at 24 different points 256 times each at 4 cm^{-1} resolution. The reference spectrum was developed by dissolving a small quantity of CHX dihydrochloride in distilled water followed by spreading and drying as a thin layer on the IR slide and scanning at a single point (n=128, 4 cm^{-1} resolution). All the spectra were derived and analysed using the OPUS software (Bruker Optic Inc., Billerica, MA).

3.4. Results

3.4.1. CLSM analysis of CHX-treated *D. acidovorans* biofilms

CLSM was used to visualize the baseline biofilm structure, thickness and cell viability in the presence (10 and 30 μg ml^{-1}) and absence of CHX. Both the CHX-tolerant WT15 and CHX-sensitive MT51 strains attached to the glass surfaces of flow cells and developed into biofilms within 24 h in the absence of CHX, after which they were exposed to CHX. The average thickness of untreated MT51 biofilms after 48 h was 31.3 ± 9 μm compared to 16.33±4.3 μm for the untreated WT15 biofilms **(**Table 3.1**)**. When the 24 h WT15 and MT51 *D. acidovorans* biofilms were treated with TSB supplemented with 10 μg ml^{-1} CHX for an additional 24 h, the mutant biofilm thickness was significantly ($P<0.05$) reduced to 13.2 μm; whereas, the wild type biofilm increased in thickness by 2 μm. When a concentration of 30 μg ml^{-1} CHX was applied for the same exposure period, the WT15 biofilm ceased to grow whereas MT51 biofilm cells were virtually all detached from the glass surface and washed out.

Table 3.1 Thicknesses of treated (10 and 30 μg ml^{-1} CHX) and control WT15 and MT51 *D. acidovorans* biofilms as determined using CLSM. Values are the mean (±SD) of 3 separate experiments. Values followed by the same letters are not significantly different at $P < 0.05$.

Biofilm type	Average thickness (μm)
WT15 control	16.33±4.3 ^{B}
WT15 treated (10 μg ml^{-1})	18.0±6.4 ^{B}
WT15 treated (30 μg ml^{-1})	16.0±3.3 ^{BC}
MT51 control	31.3±9.0 ^{A}
MT51 treated (10 μg ml^{-1})	13.2±7.0 ^{CD}
MT51 treated (30 μg ml^{-1})	8.36±3.9 ^{D}

Differences were also seen in the architecture of CHX-treated and untreated biofilms. Figs. 3.1**A** and 3.1**C** show typical CLSM images of 48 h control WT15 and MT51 biofilms, respectively; the WT strain grew as spatially-isolated, individual microcolonies consisting of relatively densely-packed cells. In accordance with this heterogeneous architecture, the thickness of the WT15 biofilm was variable throughout the flow channel. In contrast, the MT51 biofilm consisted of uniformly distributed cells enclosed within an extensive extracellular polymeric substance (EPS) matrix (31.3±9.0 μm). Following 24 h exposure to CHX at 10 μg ml^{-1} CHX, the biofilm matrices appeared similar in both cases; the WT15 biofilms underwent a shift in microcolony structure and became more homogenously distributed like the MT51 biofilms (Figs. 3.1B and 3.1D). Individual cells within the biofilm could be more clearly seen after MT51 biofilms were exposed to CHX and they appeared to be less dense than the WT15 biofilms.

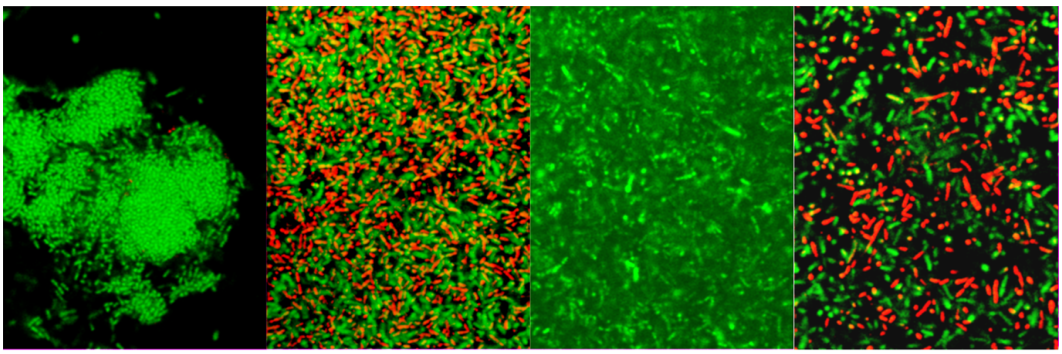

Figure 3.1 CLSM images obtained from the 5 µm sectioning depth of 48 h treated (10 µg ml^{-1} CHX) and untreated WT15 and MT51 *D. acidovorans* biofilms stained with BacLight Live/Dead stain. Biofilms were grown for 24 h using 1% TSB and then cultivated for an additional 24 h in the presence or absence of 10 µg ml^{-1} CHX. (A) WT15 control, (B) WT15 treated, (C) MT51 control, and (D) MT51 treated biofilms. Green represents viable cells; red non-viable cells.

SYTO9 and propidium iodide staining was used to determine the proportion of live and dead cells in WT15 and MT51 biofilms grown in the presence and absence of CHX. The vertical distribution of living cells within the first 20 µm of the biofilms after 48 h growth is illustrated in Fig. 3.2. Untreated biofilms consisted of 98-99% viable cells throughout the vertical profile in the case of both WT15 and MT51 biofilms. However, the mean viability of both WT15 and MT51 biofilms decreased following 10 and 30 µg ml^{-1} CHX treatment relative to untreated control biofilms. In the presence of 10 µg ml^{-1} CHX the proportion of viable cells in the WT15 biofilm increased with distance from the attachment surface over the entire thickness examined, in agreement with an increase in overall biofilm thickness, as illustrated in Table 3.1. In contrast, no definite pattern was observed for the WT15 biofilms exposed to 30 µg ml^{-1} CHX treatment. In the case of the MT51 strain, total viable cells decreased significantly ($P<0.05$) with depth following both 10 and 30 µg ml^{-1} CHX treatment compared to the MT51 control biofilm.

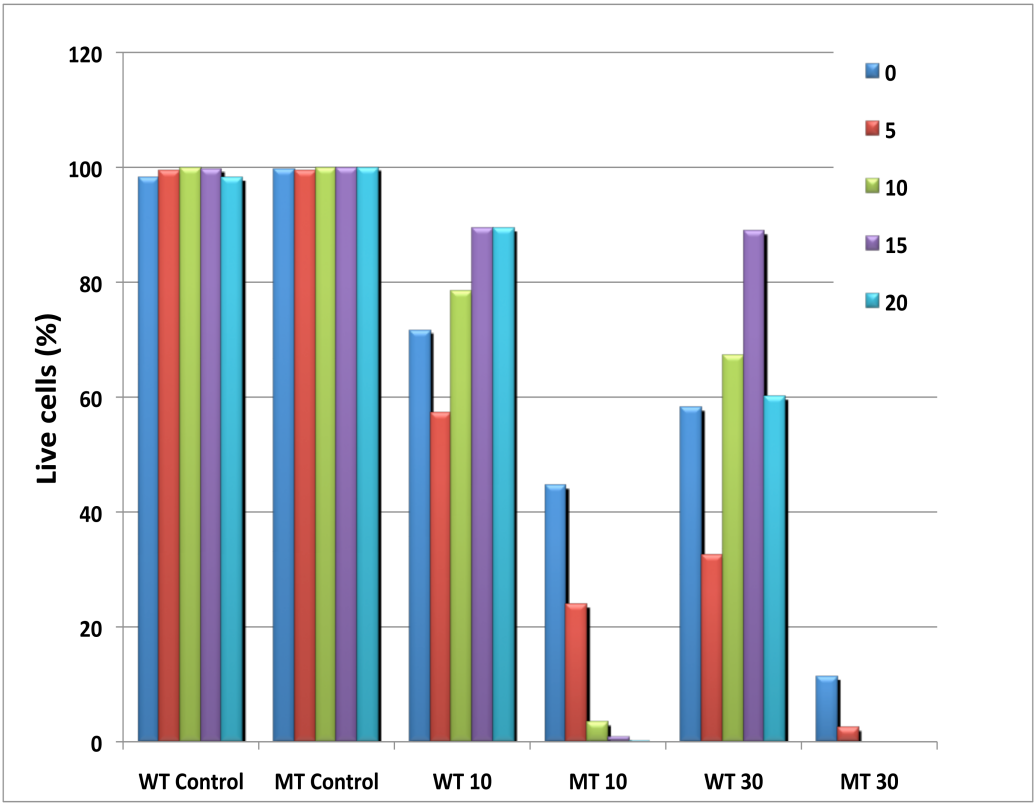

Figure 3.2 Viability profiles of 48 h WT15 and MT51 *D. acidovorans* biofilms grown for the last 24 h in the presence or absence of CHX (10 and 30 µg ml^{-1}). Determinations were made by analyzing CLSM optical thins sections of *Bac*Light Live/Dead stained-biofilms obtained at the 0, 5, 10, 15 and 20 µm sectioning depth (where 0 µm = attachment surface).

3.4.2. Soft X-ray scanning transmission microscopy (STXM)

The reference spectra used to derive the quantitative component maps for CHX and the major macromolecules in *Delftia acidovorans* biofilms, including protein, lipids, polysaccharides, carbonate, and K, have been published previously (Dynes *et al.,* 2006) and discussed in detail (Dynes *et al.,* 2009; Lawrence *et al.,* 2003; del Mar Ojeda-Vargas *et al.,* 1999). STXM images were collected at 288.2 eV for the MT51 and WT15 biofilms exposed to 0 (control) and 10 µg ml^{-1} CHX for 12 h (after 12 h of initial growth without CHX) (Fig. 3.3). Since bacteria are roughly composed of 50% protein, this photon energy is useful for preferentially visualizing bacterial cells, as it is mainly due to the absorption of the carbonyl moiety in the peptide band ((NH_2)-C=O) of proteins (Dynes *et al.,* 2006). The optical density is indicated by the gray scale, with white and black, corresponding to

higher and lower thicknesses of protein respectively. In the absence of CHX, the protein in the WT15 cells was not evenly distributed as evidenced by the wide variation in the optical density (Fig. 3.3A); whereas, there was no variation in the optical density of the cells/protein in the MT51 control system, with all being white (i.e., thickest) (Fig. 3.3B). Exposing the WT15 biofilms to CHX resulted in some of the cells having greater amounts of protein (Fig. 3.3C), suggesting that there were two different cell types, based on their response to CHX. There was no apparent change in the optical densities of the MT51 cells when exposed to CHX (Fig. 3.3D). Fig. 3.4 presents the quantitative protein, lipid and polysaccharide component maps derived from spectral fitting of 24 h-old WT15 control biofilms (obtained from the same region as for the 288.2 eV image, Fig. 3.3A). The gray scale shown in the component maps are a measure of the component thickness in nm, with the lower (black) and upper (white) limits of the scale shown for each component. The color-coded composite image (Fig. 3.4D) shows the spatial correlation of the components. No CHX signal was detected in the control biofilms. There was variation in the protein, lipids and polysaccharides content within individual cells, consisting of areas with relatively high and low content.

The quantitative protein, lipids, polysaccharides and CHX component maps for the 24 h-old WT15 biofilms exposed to 10 μg ml^{-1} CHX for 12 h (after 12 h of initial growth without CHX) are shown in Fig. 3.5. From the protein component map there were cells whose protein was unevenly distributed similar to that of the control biofilm (Figs. 3.4A,D), however, there were also cells where the protein was higher in concentration and was evenly distributed (see arrows, Fig. 3.5A). This suggests that there were two cell types in these WT15 biofilms. The cells with high protein levels also had high lipid and polysaccharide content but there was variation in their lipid and polysaccharide levels within the cells. In contrast, the lipid and polysaccharide content was fairly evenly distributed in the cells with the low lipid and polysaccharide levels. All CHX-treated cells contained CHX, with the highest level of CHX contained by cells with high protein, lipid and polysaccharide levels. Moreover, the spatial pattern of CHX distribution in the cells with high CHX content was similar to that of the lipid and polysaccharide spatial pattern.

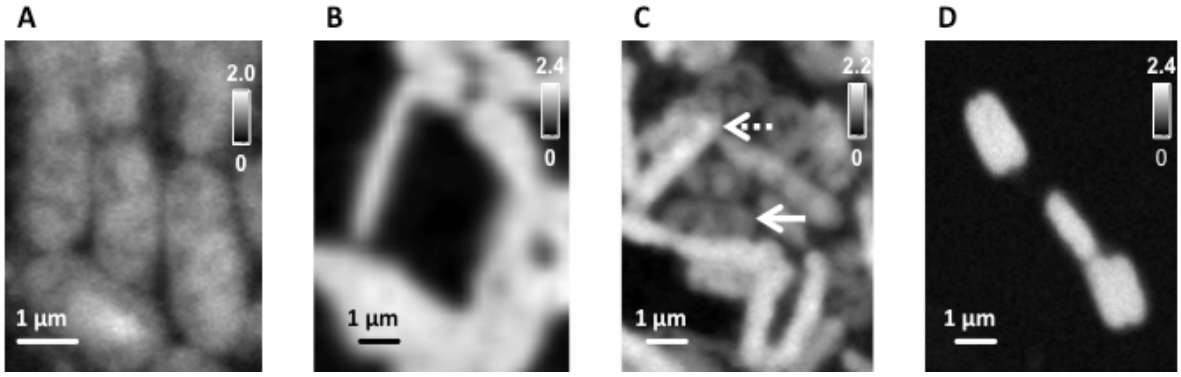

Figure 3.3 Optical density images (288.2 eV) represents the carbonyl of the protein in microbial cells) of CHX-treated (10 µg ml^{-1} for 12 h) and control biofilms of CHX-tolerant (WT15) and CHX-sensitive (MT51) *D. acidovorans* strains after 24 h of growth. (A) WT15 control, (B) MT51 control, (C) WT15 CHX-treated, and (D) MT51 CHX-treated. Arrows indicate the two cell types observed in the biofilms as evident from the density/ distribution of the protein in the cell. The gray scales indicate optical density.

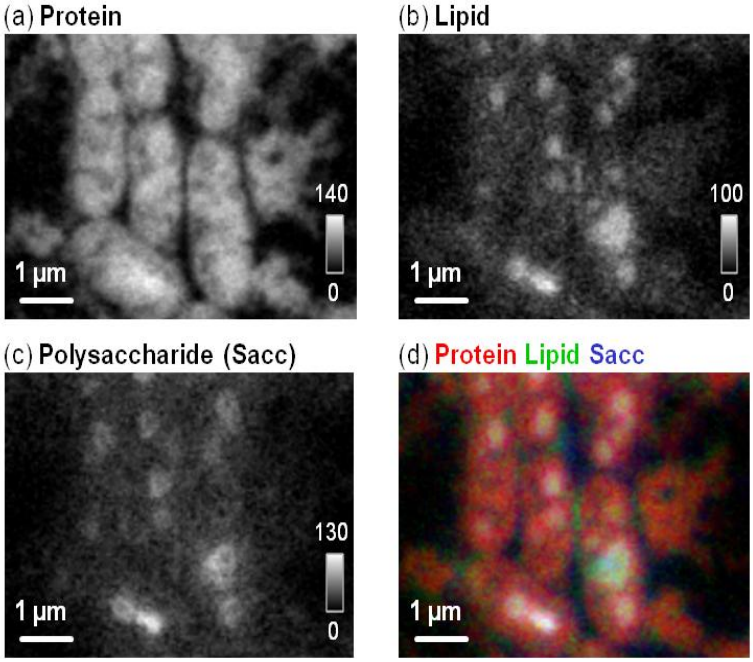

Figure 3.4 Component maps of 24 h CHX-tolerant (WT15) *D. acidovorans* biofilms not exposed to CHX. (A) Protein, (b) Lipid, and (c) Polysaccharide (Sacc). Maps (A–C) were derived by singular value decomposition (SVD) of an image sequence (280–320 eV). The gray scales indicate the effective thickness of the mapped component in nm. Color-coded composites of selected component maps (D) Protein=red, lipid=green, polysaccharide=blue.

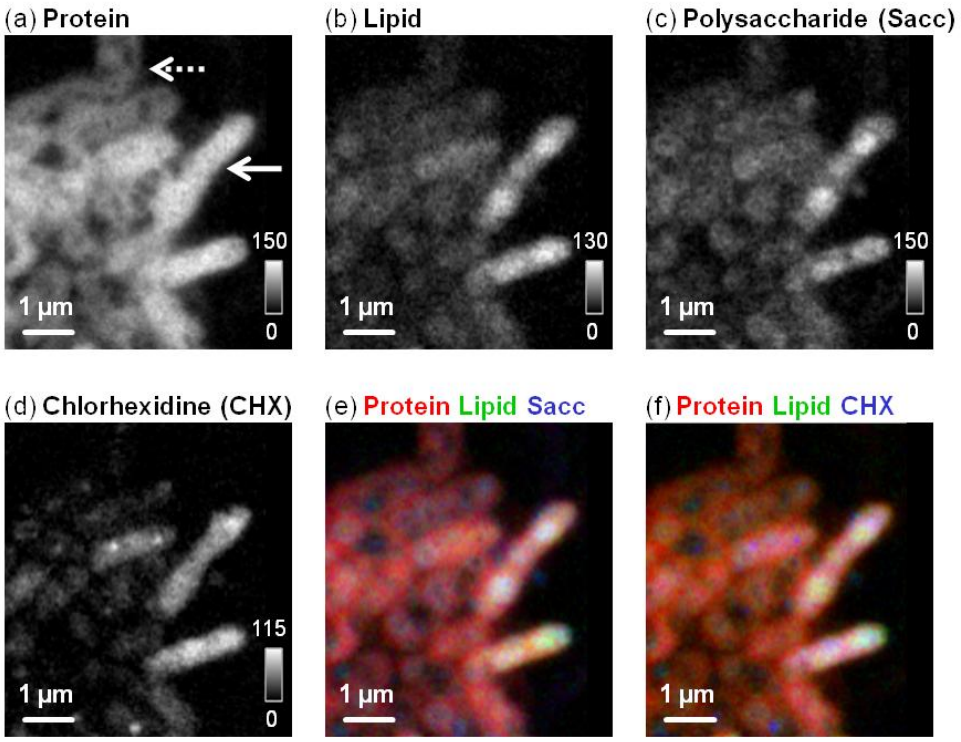

Figure 3.5 Component maps of 24 h CHX-tolerant (WT15) *D. acidovorans* biofilms treated with CHX (10 µg ml^{-1} for 12 h). (A) Protein, (B) Lipid, (C) Polysaccharide (Sacc), and (D) Chlorhexidine (CHX). Maps (A–D) were derived by singular value decomposition (SVD) of an image sequence (280–320 eV). The gray scales indicate the effective thickness of the mapped component in nm. Color-coded composites of selected component maps (E) Protein=red, lipid=green, polysaccharide=blue, and (F) protein=red, lipid=green, polysaccharide=CHX. The arrows in (A) indicate the two cell types observed in the biofilm.

The quantitative protein, lipids, polysaccharides and CHX component maps for the 24 h-old MT51 biofilms exposed to 10 µg ml^{-1} CHX for 12 h (after 12 h of initial growth without CHX) are shown in Fig. 3.6**.** Only one cell type, with even distribution of CHX, protein and lipid but not polysaccharide, was observed in all cells. The spatial distribution of CHX was different from WT15-treated cells, as in this case the pattern was similar to that of protein and lipid, rather than lipid and polysaccharide as seen in WT15 cells. The spectral curve fitting of CHX (Fig. 3.6) was performed by extracting a CHX-rich region from the image sequence to include only pixels with a value of more than 100 nm. The numerical values extracted from the spectral fit data were protein (albumin)- 95.3 nm, lipid ((1,2-dipalmitoyl-sn-glycero-3-phosphocholine)- 55.3 nm, polysaccharide (xanthan gum)- 42.0 nm, and CHX-135 nm.

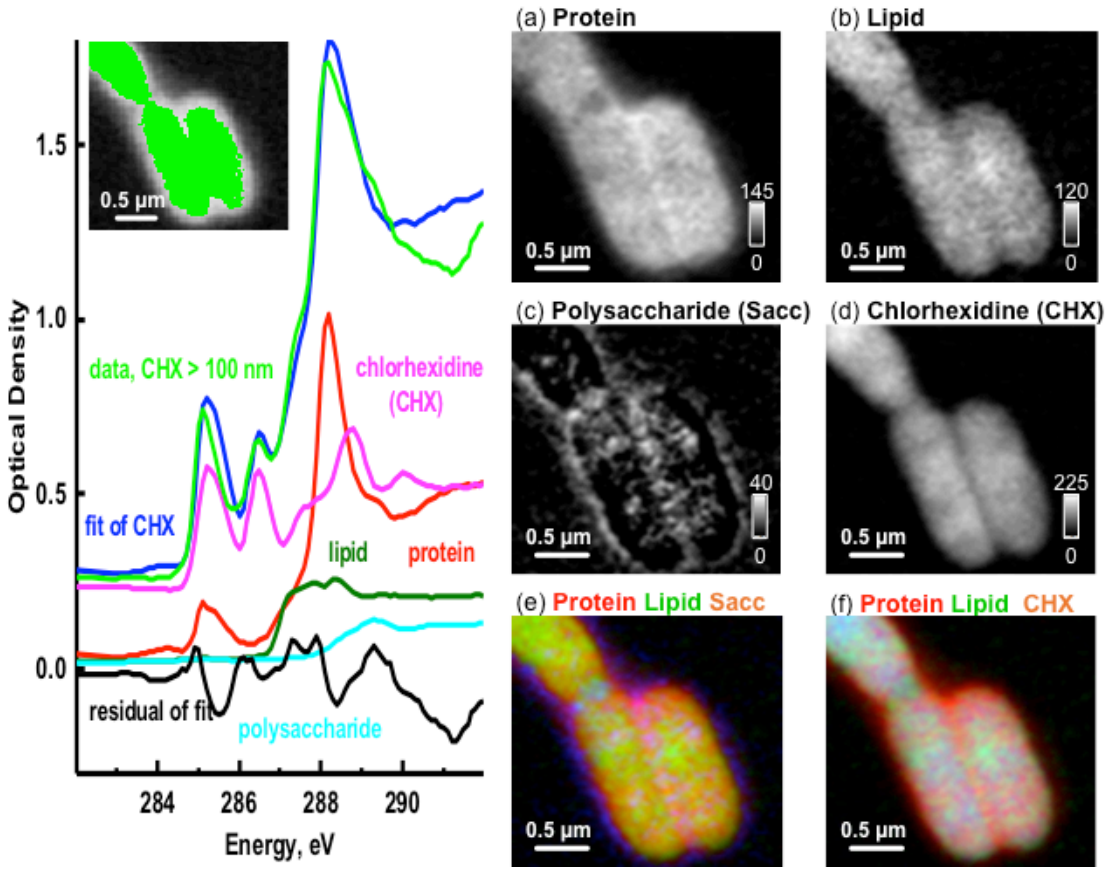

Figure 3.6 Component maps of 24 h CHX-sensitive (MT51) *D. acidovorans* biofilms treated with CHX (10 µg ml^{-1} for 12 h). (A) Protein, (B) Lipid, (C) Polysaccharide (Sacc), and (D) Chlorhexidine (CHX). Maps (A–D) were derived by singular value decomposition (SVD) of an image sequence (280–320 eV). Color-coded composites of selected component maps (E) Protein=red, lipid=green, polysaccharide=blue, and (F) protein=red, lipid=green, polysaccharide=CHX. The gray scales indicate the effective thickness of the mapped component in nm. Curve fit to the spectrum extracted from CHX component map with pixels having values more than 100 nm is also shown.

The levels of CHX in the cells from 10 µg ml^{-1} treated WT15 and MT51 biofilms were then compared (Figs. 3.5 and 3.6), and the WT15 cells accumulated CHX to a maximum thickness value of 115 nm. Whereas, CHX-exposed MT51 cells accumulated CHX to a thickness of almost twice this value. This finding is further supported by the concentration values calculated on a per pixel basis, as presented in Table 3.2, where the 10 µg ml^{-1} CHX-treated MT biofilms accumulated five times (94.1 ±58.3 nm) more CHX than the 10 µg ml^{-1} CHX-treated WT biofilms.

Table 3.2 Average thickness values (in nm) of CHX in WT15 and MT51 CHX-treated biofilms as determined from STXM images. All treatments were carried out on 12 h established biofilms.

Biofilm	**CHX nm per pixel (± Standard Deviation)**
WT15 (10 μg ml^{-1} for 12 h)	17.4 ± 19.6
MT51 (10 μg ml^{-1} for 12 h)	94.1 ± 58.3
EDTA exposed WT15 (10 μg ml^{-1} for 12 h)	36.5 ± 34.8
WT15 (30 μg ml^{-1} for 1 h)	17.2 ± 21.2
MT51 (30 μg ml^{-1} for 1 h)	58.9 ± 43.3
WT15 (30 μg ml^{-1} for 12 h)	73.2 ± 42.3
MT51 (30 μg ml^{-1} for 12 h)	101.0 ± 52.5

The effect of higher concentrations of CHX was studied only for the CHX-tolerant, WT15 *D. acidovorans* biofilms by exposing 12 h-old WT15 biofilms to 30 and 100 μg ml^{-1} of CHX for 30 and 60 min. In agreement with results obtained at lower concentration at 10 μg ml^{-1}, two different cell variants were seen at both CHX concentrations, as well as after both exposure times. However, the majority of the cells appeared lighter in the biofilms treated with 100 μg ml^{-1} CHX (Fig. 3.7) than those treated with 30 μg ml^{-1} for one hour.

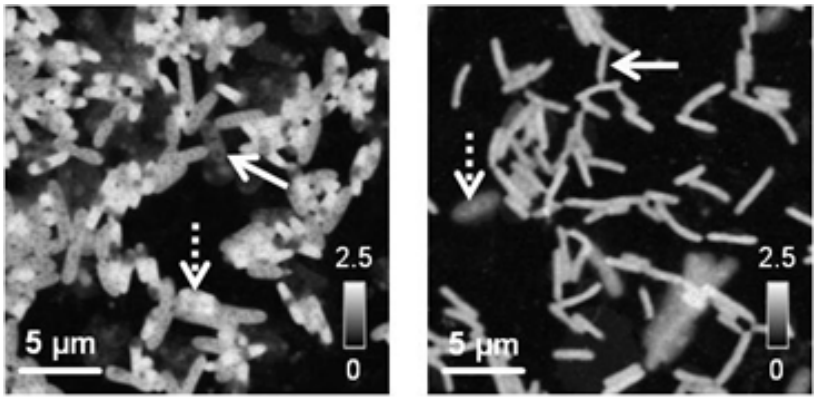

Figure 3.7 Optical density images (288.2 eV, represents the carbonyl of the protein in microbial cells) of CHX-tolerant (WT15) *D. acidovorans* biofilms grown for 12 h before treatment with (A) 30 µg ml^{-1} and (B) 100 µg ml^{-1} CHX for 1 h. The arrows indicate the two cell types observed in the biofilm.

Based on the above results, the effect of short (1 h) and long (12 h) CHX (30 µg ml^{-1}) exposure times was evaluated on both 12 h WT15 and MT51 biofilms. Fig. 3.8 shows a time-dependent effect, wherein the MT51 cells accumulated more CHX over time relative to CHX-tolerant WT15 cells. In comparison, CHX intensity (blue color) was less in CHX-tolerant cells even after 12 h of exposure. This observation was further substantiated by Table 3.2 which quantifies the differences in CHX accumulation between CHX-sensitive and CHX-tolerant cells exposed to above-MIC (30 µg ml^{-1}) CHX concentrations at both time intervals. Interestingly, both cell variants, described earlier, were seen in 1 h treated WT15 and MT51 biofilms but only the dark/dense cells were seen after long term exposure (12 h) to CHX. The 1 h STXM images were further examined in detail and showed that the denser cells had higher levels of CHX than the lighter cells within the same biofilm for both strains (Fig. 3.9 and Table 3.3). There was not much difference in CHX concentration among the light cells in both cases, but the darker cells within the MT51 CHX-treated biofilms had nearly twice as much CHX as their WT counterparts (Table 3.3). There was variation in concentration of other macromolecules as well between the dark and light cells in both WT15 and MT51 biofilms (Table 3.3), with denser cells containing primarily more protein followed by lipid and polysaccharide. The spatial distribution of CHX in 1 h CHX-exposed cells (Fig. 3.8) indicated that CHX was

initially associated with the lipid component of the cells; however, this effect was difficult to confirm with increasing CHX-exposure time, especially for the MT51 biofilms.

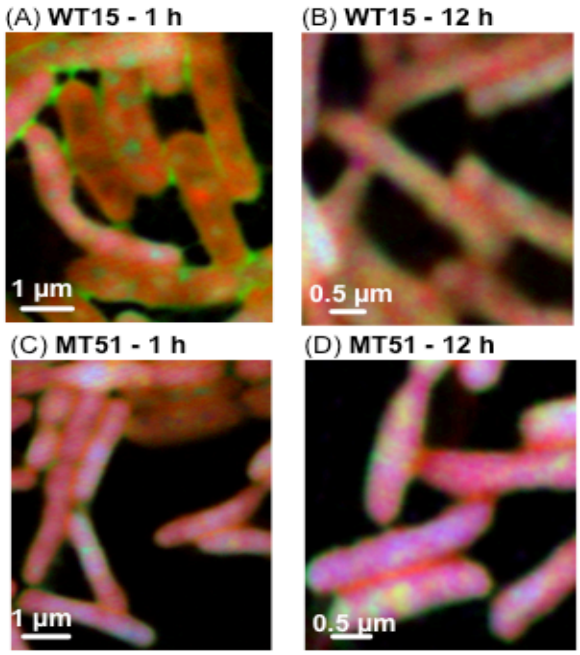

Figure 3.8 Color coded composites (protein=red, lipid=green, CHX=blue) of CHX-tolerant (WT15) and CHX-sensitive (MT51) *D. acidovorans* biofilms grown for 12 h before treatment with 30 µg ml^{-1} CHX for 1 h (A and C) and 12 h (B and D), respectively.

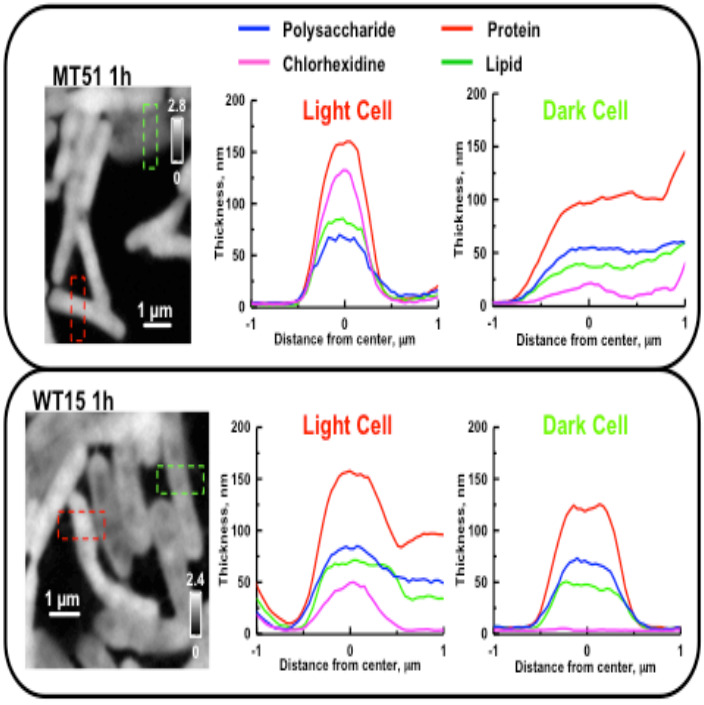

Figure 3.9 Intensity profiles extracted from a section of the dark and light microbial cells from the protein (red), lipid (green), polysaccharide (blue) and CHX (pink) component maps from Figure 8 of the WT15 and MT51 *D. acidovorans* biofilms grown for 12 h before treatment with 30 μg ml^{-1} CHX for 1 h. The figures are 288.2 eV transmission images. The red and green rectangles show the area from which the profiles for the dark and light cells were taken, respectively. The gray scales indicate optical density.

Table 3.3 Average thickness values (in nm) of major macromolecules of the two cell types of 12 h established WT15 and MT51 biofilms exposed to 30 μg ml^{-1} CHX for 1 h, as determined from STXM images.

Cell type	**Protein**	**Lipid**	**Saccharide**	**CHX**
	nm per pixel (± Standard Deviation)			
WT15-dense	148.0 ±19.3	69.7 ± 13.6	75.2 ± 18.0	45.5 ± 14.2
WT15-light	111.0 ± 20.2	45.1 ± 12.6	62.2 ± 15.0	6.92 ± 7.34
MT51-dense	141.0 ± 28.9	72.2 ± 20.4	61.4 ± 13.6	107.0 ± 30.9
MT51-light	95.5 ± 15.9	38.4 ± 9.28	52.3 ± 9.65	15.0 ± 8.61

To further investigate CHX accumulation effects, WT15 biofilms were first exposed to the membrane permeabilising agent, ethylenediaminetetraacetic acid (EDTA), before exposure to 10 μg ml^{-1} CHX. Figs. 3.10 and 3.11 show the component maps of WT15 biofilm cells exposed to EDTA only and EDTA followed by CHX treatment. As shown in Table 3.2, treatment with EDTA promoted bioaccumulation of CHX (almost double) in WT15 biofilms (36.5 ± 34.8 nm per pixel) than WT15 biofilms treated only with CHX (17.4 ± 19.6 nm per pixel). Furthermore, only one type, the darker cells that accumulated CHX in our earlier studies, were found to be present in the EDTA treated biofilms (Fig. 3.11).

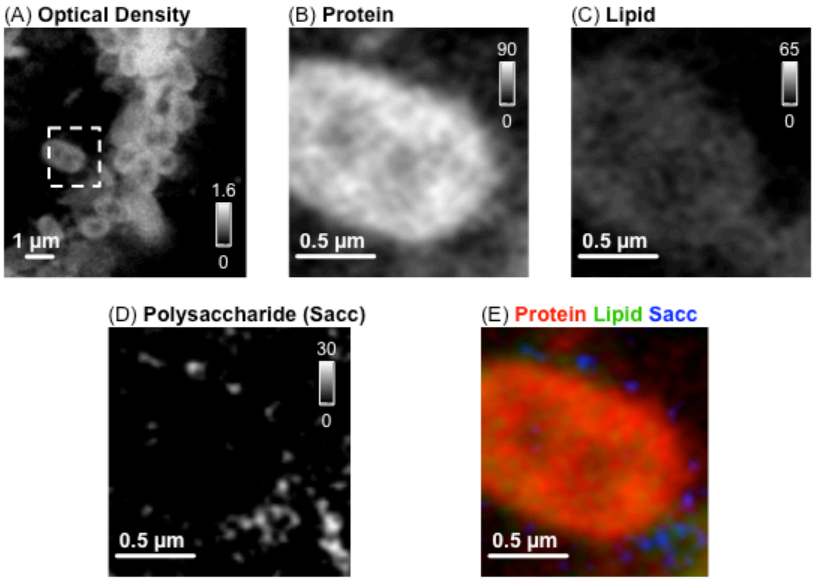

Figure 3.10 CHX-tolerant (WT15) *D. acidovorans* biofilms treated with 1 mM EDTA for 24 h. (A) Optical density (OD) image (288.2 eV). The gray scale indicates OD. The white dotted rectangle shows the microbial cell studied in detail. Component maps of the microbial cell (B) Protein, (C) Lipid and (D) Polysaccharide (Sacc) and a color-coded composite (E) of the selected component maps (protein=red, lipid=green, polysaccharide=blue). Maps (B–D) were derived by singular value decomposition (SVD) of an image sequence (280–320 eV). The gray scale (B-D) indicates the effective thickness of the mapped component in nm. EDTA could not be detected in, or near, the microbial cell.

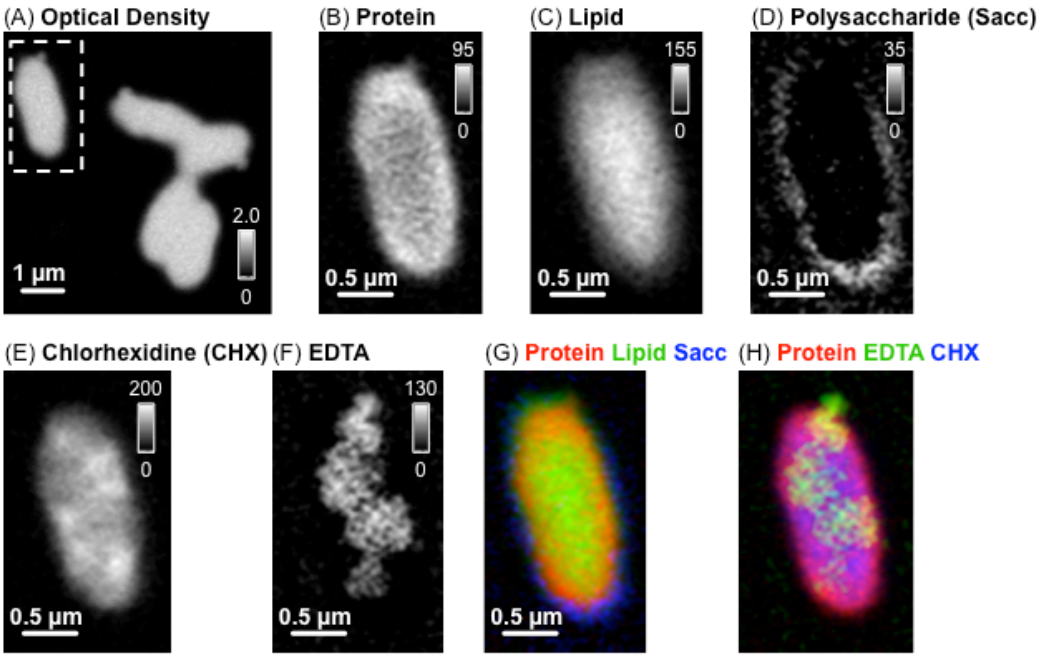

Figure 3.11 CHX-tolerant (WT15) *D. acidovorans* biofilms treated with 1 mM EDTA and 10 μg ml^{-1} CHX. (A) Optical density (OD) image (288.2 eV). The gray scale indicates OD. The white dotted rectangle shows the microbial cell studied in detail. Component maps of the microbial cell (B) Protein, (C) Lipid, (D) Polysaccharide (Sacc), (E) CHX, and (F) EDTA. Maps (B–D) were derived by singular value decomposition (SVD) of an image sequence (280–320 eV). The gray scale (B-D) indicates the effective thickness of the mapped component in nm. Color-coded composites of the selected component maps (G) Protein=red, Lipid=green, Polysaccharide=blue, and (H) Protein=red, EDTA=green, CHX=blue.

3.4.3 Infrared Spectroscopy

Specific IR absorption patterns can be attributed to particular types of covalent bonds and any modifications of these bonds by the local environment can be detected in the details of the IR spectra. Accordingly, vibrational signatures of organic molecules in microbial biofilms can be measured using IR spectromicroscopy. The main absorption bands of biofilms correlating with the experimental conditions employed in this study are plotted in Fig. 3.12A**,** with the IR spectrum of the CHX standard provided for reference. The subset panel of IR spectra reveals a peak at around 1492 cm^{-1} which is attributed to the chlorophenol groups of CHX (Holman *et al.,* 2009). Two peaks at the 3400 cm^{-1} region were also seen in the CHX IR spectrum and could be attributed to $\upsilon\upsilon_{as}$ and $\upsilon\upsilon_{as}$- NH, which is also suggestive of CHX (Cortes *et al.,* 2001). Peaks were also observed in 1650, 1600, 1550 and 1500 cm^{-1} that can be attributed to the C=C stretching of the aromatic moiety of CHX (Cortes *et al.,* 2001). The peak at 1492 cm^{-1}, enlarged in Fig. 3.12B**,** was very prominent in CHX-sensitive, MT51 biofilms treated with 10 and 30 μg ml^{-1} CHX, with the peak area being larger in biofilms receiving the latter treatment. In contrast, the CHX diagnostic peak at 1492 cm^{-1} was absent in the control biofilms without CHX.

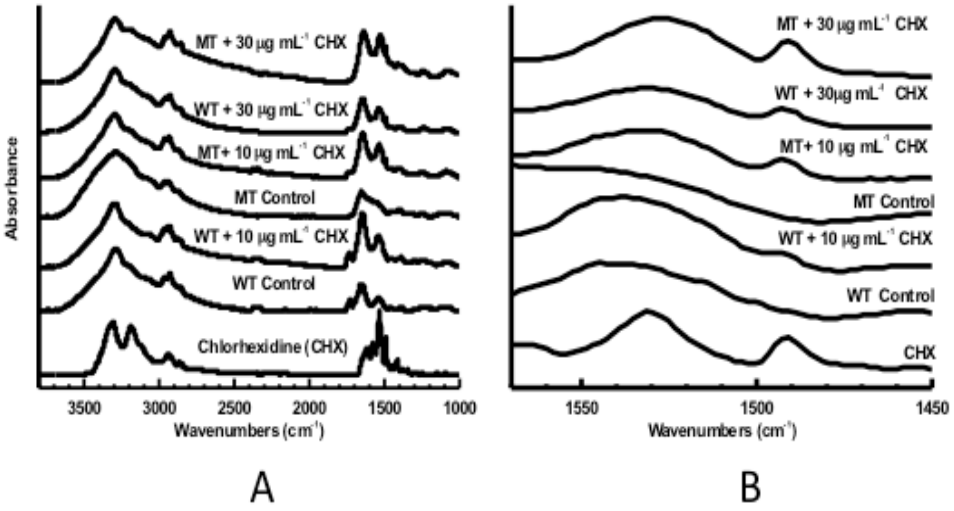

Figure 3.12 IR spectra obtained for WT15 and MT51 *D. acidovorans* biofilms not exposed to CHX for 48 h and CHX-treated WT15 and MT51 *D. acidovorans* biofilms (10 and 30 µg ml^{-1} for 24 h). (A) 1000 to 4000 cm^{-1} and (B) expansion of (A) from 1450 to 1600 cm-^{1}. WT15 and MT51 control biofilms multiplied by 5 and CHX treated biofilms multiplied by 2.

3.5. Discussion

Association of *Delftia* spp. with antibiotic resistance phenomena has been referenced in the literature but overall is very limited (Ravaoarinoro and Therrien, 1999; Chotikanatis *et al.,* 2011; Stampi *et al.,* 1999). To our knowledge, this is the first report of *D. acidovorans* tolerance to a broad spectrum biocide such as CHX. Several strains of *C. acidovorans* isolated from clinical specimens have been found to be resistant to various groups of antibiotics such as β-lactams, aminoglycosides and quinolones (Ravaoarinoro and Therrien, 1999). Chotikanatis *et al*. (2011) summarized the antibiotic susceptibilities of *D. acidovorans* reported in the past and concluded that all were resistant to gentamicin, some to all aminoglycosides and generally susceptible to cephalosporins, piperacillin, aztreonam, carbapenems, quinolones, and trimethoprim-sulfamethoxazole. A positive correlation between *C. acidovorans* and residual chlorine was found in contaminated dental water units, indicating a strong resistance of this bacterium to chlorine (Stampi *et al.,* 1999). Additionally, a *D. acidovorans* strain isolated from a wastewater treatment facility was found to carry class 3 integrons, genetic elements commonly-associated with antibiotic

resistance genes. This finding added a new role for this group of organisms in the acquisition of resistance genes from antibiotic-resistant microbes in the environment (Xu *et al.,* 2007).

The MIC levels for CHX have been tested for many bacterial species and the range of tolerance is very large and dependent on factors such as type of bacteria (Gram-positive or Gram-negative), method or media used, etc. In a recent study (Valenzuela *et al.,* 2013), CHX exhibited the greatest variability with MIC's in a range from 2.5 to 2,500 mg ml^{-1} when 272 Enterococcus strains were tested for sensitivity to biocides of different classes (quaternary ammonium compounds, a bisphenol, a biguanide and copper sulfate). Similarly, the MIC of CHX for 52 strains of subgingival plaque (biofilm) bacteria ranged from 8 to 500 μg ml^{-1} (Stanley *et al.,* 1989); whereas, for methicillin-resistant *Staphylococcus aureus* isolates collected over 15 years, the MIC range was found to be 0.5 to 16 μg ml^{-1} (Wang *et al.,* 2008). The MIC of four common human pathogens, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* were 0.008, 0.0625, 0.125 and 0.002 μg ml^{-1}, respectively (Houari and Martino, 2007), and MICs varied between 1-20 μg ml^{-1} for various Gram-positive and Gram-negative oral bacteria examined (Susi *et al.,* 2001). Based on comparison of the chlorhexidine MIC value for WT15 *D. acidovorans* (15 μg ml^{-1}) with MIC values from other reported studies, WT15 can be considered as "tolerant" to CHX.

While both the WT15 and MT51 *D. acidovorans* strains used in this study were demonstrated to form biofilms, the MT strain formed thicker biofilms than the WT in the absence of CHX. The biofilm structure, in terms of cell distribution and density, was also different between WT15 and MT51 strains (Fig. 3.1). The reason for this difference may be related to fitness costs associated with CHX tolerance as a result of the Tn5 mutation. Previous studies have demonstrated that the cost associated with chromosomal resistance will vary profoundly depending on the growth and experimental conditions used (Andersson and Levin, 1999). The relative fitness of susceptible and resistant bacteria is measured by their reproductive success during *in vitro* culture. Many studies have reported that resistance commonly creates a fitness burden (Andersson and Levin, 1999; Andersson and Hughes, 2010); however, resistant mutants with no measurable costs or those with a slight advantage over wild

type have also been observed (Bjorkman *et al.,* 1998; Miskinyte and Gordo, 2013), which is in agreement with our observations. However, in the presence of CHX at 10 μg ml^{-1}, the biomass of the WT15 biofilms increased over a 24 h period; whereas, there was an apparent cessation of biofilm development in the CHX-treated MT51 biofilms compared to their untreated counterparts. It is also possible that exposure of MT51 cells to CHX could result in dispersal of biomass contributing to an apparent reduction in biofilm thickness of MT51 biofilms. Such stimulation of biofilm development, as seen in the WT15 biofilm in the presence of sub-MIC concentration of CHX, has previously been reported (Hope and Wilson, 2004). In addition to the change in overall biofilm thickness observed in our study, the form and arrangement of microcolonies in both types of biofilms became altered upon exposure to CHX. The cells in control WT15 biofilms were arranged in very close association with each other, but upon exposure to CHX, these distinct microcolonies ceased to be evident. Hope and Wilson (2004) observed biofilm contraction when 0.2% CHX was used on plaque biofilms (Hope and Wilson, 2004), attributing this to ionic interactions between the negatively-charged EPS matrix and the positively-charged CHX molecules. They reported that these interactions changed the EPS solubility, hydrophobicity and net charge of the matrix, thus reducing the repulsive forces between charged moieties and allowing the biofilm matrix to contract. However, at 0.05% CHX exposure, the contraction was not significant and non-uniform and hence they concluded that biofilm contraction may be related to the concentration of CHX as well.

Since CHX effects include bacterial membrane damage (Gilbert and Moore, 2005; Vitkov *et al.,* 2005; Barrette-Bee *et al.,* 1994), its bactericidal action can be measured using the *Bac*Light LIVE/DEAD stain (Hope and Wilson, 2004, Dynes *et al.,* 2009; Shen *et al.,* 2009; von Ohle *et al.,* 2010) that is sensitive to the permeability of the cytoplasmic membrane. Upon CHX treatment the percentage of viable cells, based on maintaining selective membrane permeability, decreased in both WT15 and MT51 biofilms (Fig. 3.2) to varying degrees. The proportion of viable cells in the WT15 biofilm increased in the presence of 10 μg ml^{-1} CHX from the base of the biofilm (the attachment surface) to biomass located further away from the attachment surface (and closest to the bulk liquid phase containing CHX). This is in agreement with an increase in biofilm thickness in the presence of CHX (Table 3.1). In contrast to WT15 biofilms**,** a gradual loss of green fluorescence, indicative of a reduction of "living cells"

was observed beginning at the biofilm-liquid interface in MT51 biofilms exposed to CHX. MT51 cells in direct contact with CHX were affected by the biocide to a greater extent than cells located deep within the biofilm matrix. These affected cells may have subsequently become detached from the biofilm base, an occurrence consistent with the observed reduction in biofilm thickness in MT51 biofilms upon CHX treatment. These results are also in agreement with other studies (von Ohle *et al.,* 2010; Pratten *et al.,* 1998; Takenaka *et al.,* 2008) where similar patterns of viability were observed in biofilm cells exposed to CHX. However, we did not see a complete eradication of the biofilms at either concentration of CHX (10 μg ml^{-1} or 30 μg ml^{-1}) used in this study. Interestingly, low numbers of MT51 cells continued to persist even at 30 μg ml^{-1}, a value thirty times the MIC for planktonic cells (1 μg ml^{-1}), which is in agreement with the paradigm that biofilm growth renders cells 10 to 1000 times more resistant than their planktonic counterparts (Hoiby *et al.,* 2010; Mah and Toole, 2001).

Confocal microscopy demonstrated the effect of CHX on MT51 and WT15 biofilms; however, there was a need to map the spatial distribution and uptake of CHX to understand the differences in CHX tolerance in these two strains. Dynes *et al.,* 2006 were the first to observe the bio-accumulation of CHX in lipid-rich regions of diatoms and bacteria using STXM to study CHX-exposed river biofilms. In the present study using STXM, bioaccumulation of CHX was similarly also seen in *D. acidovorans* WT15 and MT51 biofilms, although to different extents. A significant question arises as to whether all cells in the WT15 biofilm exhibited the same level of tolerance, or whether there was a sub-population that existed as "persister" cells (Lewis, 2001; Keren *et al.,* 2004) and were able to tolerate CHX. We thus suggest that the WT15 *Delftia* biofilms contains two populations (as shown in Figs. 3.3, 3.7, 3.9); those that apparently do not, or only slowly, take up CHX (light cells) and those that do (dark cells), with the former being responsible for persistence and tolerance within WT15 biofilms. STXM mapping results showed that CHX was distributed throughout the cell and could not be linked to any particular cellular macromolecule or region. As well, the MT51 biofilm cells accumulated five times the amount of CHX as did the WT15 cells, in agreement with their increased sensitivity to CHX (Table 3.2). When the WT15 biofilms were exposed to higher CHX concentrations (30 μg ml^{-1} and 100 μg ml^{-1}), the number of dark cells increased accordingly. The apparent absence of light cells in 100 μg ml^{-1} CHX-exposed WT15 biofilms indicates that

intrinsic CHX tolerance tends to decrease at CHX concentrations greater than 30 μg ml^{-1}. Temporal analysis of biofilm cells treated with 30 μg ml^{-1} also revealed that the MT51 cells accumulated more CHX than the WT15 strain over a 1 h period, indicating an immediate effect of CHX on the sensitive cells. Interestingly, examination of 1 h CHX-exposed (30 μg ml^{-1}) biofilm cells revealed the association of CHX with cellular lipids (Fig. 3.8); whereas, at lower CHX concentration (10 μg ml^{-1}) no association of CHX with any particular cellular component could be established (Fig. 3.5). This finding is consistent with other reported observations (Dynes *et al.,* 2006; Castillo *et al.,* 2004; Elferink and Booij, 1974) where associations between CHX and lipids were made. This is also consistent with the fact that CHX is a positively-charged hydrophobic, lipophilic molecule which interacts with negatively-charged lipopolysaccharide molecules of the bacterial outer membrane before being able to enter into the cell.

Our observations of the presence of two different kinds of cells within the CHX-exposed WT biofilms is supported by similar work (Tattawasart *et al.,* 2000) where energy-dispersive analysis of X-ray (EDAX) mapping revealed differences in CHX uptake between CHX-sensitive and resistant isolates of *Pseudomonas stutzeri*. In this work, X-ray fluorescence signals of chloride (Cl) increased with time and were evenly distributed throughout the entire cell in a CHX-treated sensitive strain. CHX-resistant isolates were less electron-dense and the chlorine signal increased with time from 30 to 60 min. Similarly, time-of-flight secondary-ion mass-spectrometry (TOF-SIMS) analysis was also able to distinguish between living and dead cells of *Candida albicans* biofilms that were treated with chlorhexidine digluconate (Tyler *et al.,* 2006). The dead cells did not contain high levels of intracellular potassium when compared to the living ones, indicative of a disrupted membrane. Furthermore, the chlorhexidine digluconate intensity in the dead cells was found to be higher than that in the surrounding medium, indicating that chlorhexidine digluconate bound preferentially to cellular components of the *C. albicans* biofilms. Transmission electron microscopic images of *P. aeruginosa* cells treated with 10 μg ml^{-1} CHX also revealed two cell types that correlated with a sensitive and a surviving cell type (Richards and Cavill, 1979) based on the appearance of the cell envelope.

In the present study, EDTA was shown to promote bioaccumulation of CHX in CHX-tolerant biofilms, as CHX was mapped in all of the EDTA-treated biofilm cells (wherein all cells were of the dark morphotype), which was not the case observed when the biofilms were treated with CHX alone. This further strengthens our CLSM findings that the bacterial membrane plays a key role in *Delftia* CHX tolerance. EDTA is well known to have a significant effect on the outer membrane permeability of Gram-negative bacteria (Lieve, 1974).

IR spectroscopy has been used to evaluate the bulk chemical changes taking place within microbial biofilms (Holman *et al.,* 2009; Quiles *et al.,* 2010). In our study, the results from IR spectroscopic analysis were in agreement with our STXM results, as the diagnostic IR CHX-peak at 1492 cm^{-1} was seen in all CHX-treated biofilms, but more prominently in the MT51 biofilm, particularly at high CHX concentrations (Quiles *et al.,* 2010). This is consistent with the observed increased accumulation of CHX in CHX-sensitive MT51 cells detected with STXM. Since these peaks were highly-comparable with the CHX standard, we may further conclude that CHX does not appear to be modified or degraded within *D. acidovorans* biofilms. CHX degradation studies previously conducted in our laboratory using ^{14}C-radiolabelled CHX and liquid scintillation spectrometry demonstrated that *D. acidovorans* (WT15) cannot mineralize CHX (data not shown), though CHX degradation by several other bacterial species is known (Tanaka *et al.,* 2005).

3.5.1. Conclusions

CHX is a clinically useful and important antibacterial agent; however, some Gram-negative bacteria appear to be highly-resistant to CHX by a mechanism that, as yet, is not fully understood. *Delftia acidovorans* biofilms provided an ideal model system for integrating analysis of CLSM, STXM and IR data to gain insights into bacterial interactions with CHX at the micro- to nano-scales of resolution. While there were differences in the growth responses of biofilms exposed to CHX, it was significant that the biofilm mode of growth elevated the tolerance levels (compared to the planktonic MIC values) of both CHX-tolerant and CHX-sensitive strains of *D. acidovorans*. It thus appeared that even long-term exposure to CHX was not sufficient to completely eradicate *D.*

acidovorans biofilms of either strain. STXM allowed quantitative and high resolution (~30 nm) *in situ* mapping of the spatial distribution of CHX in cells and revealed differential uptake of CHX in WT15 and MT51 *D. acidovorans*. Disruption of the membrane protein *TolQ* gene, along with differential intake of CHX by these strains and increase intake of CHX in presence of the membrane permeabilising agent, EDTA, indicates the potential role of the cell membrane in CHX resistance. Although the microscopy/spectroscopy techniques used in this study can be employed as useful tools in studying these phenomena, more detailed molecular characterization of the role of *TolQ* gene in CHX resistance in these strains will further provide insights into the actual mechanism of CHX resistance.

3.6. Connection to next study

Study 1 demonstrated the differences between the CHX-tolerant and CHX-susceptible strains of *D. acidovorans* biofilms exposed to CHX using microscopic techniques, and suggested a possible role of the cell membrane and TolQ in CHX tolerance. The following study was undertaken to further understand the molecular mechanisms of CHX tolerance in these strains primarily by employing a well-known proteomic technique (DIGE).

4. PROTEOMIC ANALYSES OF CHLORHEXIDINE TOLERANCE MECHANISMS IN *DELFTIA ACIDOVORANS* BIOFILMS

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Author contributions

All authors participated in the design of the experiments and contributed to writing of the manuscript. Preparation of the initial draft of the manuscript, as well as all other data presented in this manuscript, are the work of the thesis author.

4.1. Abstract

Protein expression and fatty acid profiles of biofilm cells of chlorhexidine-tolerant *Delftia acidovorans* (MIC = 15 μg ml^{-1}) and its chlorhexidine-susceptible mutant (MIC = 1 μg ml^{-1}) were investigated. The chlorhexidine-susceptible mutant (MT51) was derived from the parental strain (WT15) using Tn5 transposon mutagenesis. The disrupted gene was identified as *tolQ*, a component of the *TolQRAB* gene cluster, known to be involved in outer membrane stability. Proteomic responses of biofilm cells were compared by differential in-gel electrophoresis following exposure to chlorhexidine at sub-MIC (10 μg ml^{-1}) and above-MIC (30 μg ml^{-1}) concentrations. Numerous changes in protein abundance were observed in biofilm cells following chlorhexidine exposure, suggesting that molecular changes occurred during adaptation to chlorhexidine. Forty proteins showing significant differences (fold change ≥ 1.5, P <0.05) were identified by mass spectrometry and were associated with various functions, including amino acid and lipid biosynthesis, protein translation, energy metabolism and stress-related functions (e.g. GroEL, aspartyl/glutamyl-tRNA amidotransferase, elongation factor Tu, Clp protease, hydroxymyristoyl-ACP dehydratase). Several proteins involved in fatty acid synthesis were affected by chlorhexidine, in agreement with fatty acid analysis, wherein chlorhexidine-induced shifts in the fatty acid profile were observed in the chlorhexidine-tolerant cells, primarily the cyclic fatty acids. Transmission electron microscopy revealed more prominent changes in the cell envelope of chlorhexidine-susceptible MT51 cells. This study suggests that multiple mechanisms involving both the cell envelope (and likely TolQ) and pan-metabolic regulation play roles in chlorhexidine tolerance in *D. acidovorans*.

4.2. Importance

Delftia acidovorans has been associated with a number of serious infections, including bacteremia, empyema, bacterial endocarditis, and ocular and urinary tract infections. It has also been linked with a variety of surface-associated nosocomial infections. Biofilm-forming antimicrobial resistant *D. acidovorans* strains have also

been isolated, including ones displaying resistance to the common broad spectrum agent, chlorhexidine. The mechanisms of chlorhexidine resistance in *D. acidovorans* are not known; hence, a chlorhexidine-susceptible mutant of the tolerant wild type strain was obtained using transposon mutagenesis, and the proteome and ultrastructural changes of both strains were compared under chlorhexidine challenge.

4.3. Introduction

Chlorhexidine is a broad-spectrum antimicrobial agent with wide application as an active ingredient in many daily-use disinfectants, pharmaceutical, personal and healthcare products (Ranganathan, 1996). Though chlorhexidine is often referred to as a "gold-standard" agent (Jones, 1997; Moshrefi, 2002) by health professionals, owing to its antibacterial efficacy, there are several reports of microorganisms surviving in the presence of in-use chlorhexidine concentrations (Marrie and Costerton, 1991; Stickler *et al.,* 1987; Brooks *et al.,* 2002). However, very little is known of the underlying mechanisms for this tolerance. Chlorhexidine tolerance compared to their planktonic counterparts (Hoiby *et al.,* 2010). Antimicrobial resistance mechanisms in biofilm bacteria have been well-studied (Hoiby *et al.,* 2010; Lewis, 2001; Mah and Toole, 2001). However, there remains a dearth of understanding of the mechanisms involved in chlorhexidine resistance in microbial biofilms. The elucidation of the molecular details of antimicrobial resistance continues to be an active and valuable area of research. The few molecular studies conducted to date on chlorhexidine resistance have mainly relied upon genomic approaches (Fang *et al.,* 2002; Wang *et al.,* 2008; Coenye *et al.,* 2011). However, proteomic analysis has also proven valuable for antimicrobial resistance studies involving a variety of agents (Mangalapalli-Illathu and Korber, 2006; Weber *et al.,* 2008; Pinto *et al.,* 2010; Cagas *et al.,* 2011). An important distinction between genomic and proteomic approaches is that only expressed genes yielding functional proteins are detected.

Gel-to-gel variability is a major drawback of gel-based techniques, leading to problems in detecting and quantifying differences in protein expression (Asirvatham *et al.,* 2002). Ünlü *et al.,* (1997) developed an approach

involving the multiplexing of fluorescently-labeled samples on the same gel; two-dimensional difference in-gel electrophoresis (DIGE). DIGE involves pre-labelling of different protein samples with spectrally-resolvable fluorescent dyes that are charge- and mass-matched, ensuring an equivalent impact on in-gel migration of labelled proteins and improving the overall accuracy of relative quantification between samples (Karp *et al.,* 2004).

Delftia acidovorans is a Gram negative bacterium ubiquitously found in soil and water and associated with a number of serious infections, including: bacteremia, empyema, bacterial endocarditis, and ocular and urinary tract infections (del Mar Ojeda-Vargas *et al.,* 1999; Cho and Lee, 2002; Perla and Knutson, 2005). *Delftia acidovorans* has also been implicated in various nosocomial biofilm infections associated with the use of medical devices like vascular catheters (Ender *et al.,* 1996; Kawamura *et al.,* 2011), pressure-monitoring devices (Weinstein *et al.,* 1996) and surgical instruments (Mino de Kaspar *et al.,* 2000). Relatively little is known about *D. acidovorans'* pathogenicity, prevalence, health and environmental risks, genetic information, antibiotic resistance profile and stress response mechanisms. Clinical *D. acidovorans* strains have shown resistance to β-lactams and related antibiotics such as aminoglycosides and quinolones (Ravaoarinoro and Therrien, 1999, Kam *et al.,* 2012). A strain resistant to chlorine was found in contaminated dental water units (Stampi *et al.,* 1999). More recently, *Delftia* spp. were shown to carry class 3 integrons, the genetic elements commonly-associated with antibiotic resistance genes (Xu *et al.,* 2007), indicating that this organism may horizontally-acquire resistance genes from microbes in the environment.

A chlorhexidine-tolerant *D. acidovorans* strain (WT15) (MIC = 15 μg ml^{-1}) was isolated from a river biofilm and characterized (Rema *et al.,* 2014). A chlorhexidine-susceptible mutant (MT51) (MIC = 1 μg ml^{-1}) was derived from WT15 using Tn5 transposon mutagenesis (Rema *et al.,* 2014). We then used whole-proteome analysis of chlorhexidine-tolerant and chlorhexidine-susceptible *D. acidovorans* biofilms grown in the presence and absence of chlorhexidine in order to examine the adaptive response.

4.4. Materials and methods

4.4.1. Bacteria, culture conditions and MIC determination

A chlorhexidine-tolerant strain (WT15) of *D. acidovorans* (MIC = 15 μg ml^{-1}) was isolated and characterized from South Saskatchewan River water biofilms (Rema *et al.,* 2014). Resistance to biocides is often referred to as tolerance (Rema *et al.,* 2014). Based on comparison of the chlorhexidine MIC value for WT15 *D. acidovorans* (15 μg ml^{-1}) with MIC values from other reported studies, WT15 can be considered as being "tolerant" to chlorhexidine. The chlorhexidine-susceptible mutant (MT51) (MIC = 1 μg ml^{-1}) was created from WT15 using Tn5 transposon mutagenesis and previously characterized in detail (Rema *et al.,* 2014). For biofilm cultivation purposes, the wild-type and mutant strains were grown from frozen stock cultures that were stored in 10% (vol/vol) glycerol at −80°C on full strength tryptic soy agar (TSA) plates, and incubated overnight at room temperature (22 ± 2°C). Well-isolated colonies were then grown in 1% strength TSB till mid-log phase (optical density of 0.4 at 600 nm) and subsequently used to inoculate the flow cells set up for the study.

Delftia acidovorans strains were screened for antibiotic resistance by using Sensititre CMV1AGNF plates (TREK Diagnostic Systems, Thermo Scientific, Waltham, Massachusetts) and the microdilution method of Andrews (2001). The plates contained 17 antimicrobial agents dosed in 96 wells at appropriate 2-fold dilutions (i.e., 64, 32, 16, 8, 4, 2,1, 0.5, 0.25, 0.12 μg ml^{-1}) following the National Antimicrobial Resistance Monitoring System of the Centers for Disease Control. Each well of the microtiter plate was inoculated according to manufacturer's instructions, followed by room temperature incubation for 24 h. MIC's were manually determined for each isolate as the lowest concentration of each antibiotic that inhibited visible growth. MIC values for chlorhexidine were conducted using the same microdilution scheme. Subsequently, a finer set of chlorhexidine concentrations were used (i.e., 9, 10, 11, 12, 13, 14, and 15 μg ml^{-1}). The MIC breakpoints were determined according to the Clinical and Laboratory Standards (formerly the National Committee for Clinical Laboratory

Standards) M100 (NCCLS, 2002) and M31 (NCCLS, 2002a) standards.

4.4.2. Identification of transposon insertion site

The gene interrupted by transposon (Tn5) insertion in *D. acidovorans* (MT51) was identified by the rapid amplification of transposon ends (RATE) technique, as described by Karlyshev *et al.* (2000). Amplified products were analysed by electrophoresis on a 1% agarose gel in 1X TAE buffer containing 0.5 μg ml^{-1} ethidium bromide. The product band was purified using the QIAquick Gel Purification kit (Qiagen, Mississauga, ON) as per the manufacturer's instructions and then sent to the National Research Council of Canada, Saskatoon, Saskatchewan, Canada for sequencing. The sequence of the disrupted gene (*tolQ*) from WT15 was then compared with sequences published in the NCBI Nucleotide Database. The sequence data for *TolQ* was submitted to the GenBank database under accession number KT988307. TolQ has been shown to be an integral cytoplasmic membrane protein required for maintenance of the integrity of the bacterial envelope in *E. coli* (Vianney *et al.,* 1994).

4.4.3. Cultivation of biofilms

Wild type and mutant biofilms were cultivated in multichannel flow cells, as explained in Rema *et al.* (2014). To evaluate the effect of chlorhexidine, established (24 h) biofilms were treated for another 24 h period with sterile medium supplemented with either sub-MIC (10 μg ml^{-1}) or above-MIC level (30 μg ml^{-1}) chlorhexidine concentrations. Biofilms that were not exposed to chlorhexidine and grown under similar conditions for a period of 48 h served as controls.

4.4.4. Transmission Electron Microscopy (TEM)

Biofilm cells were aseptically scraped from flow cells and the material transferred directly to sterile

microcentrifuge tubes. The tubes were couriered on ice to the Electron Microscope Facility, McMaster University, ON, where further processing of the samples was immediately performed, as per the procedure followed by Lawrence *et al.* (2003) with a few modifications. Ultra-thin sections were cut with a diamond knife mounted on a Leica UCT Ultramicrotome and placed on TEM grids (Marivac Canada, Canton de Gore, PQ). Lastly, sample thin-sections were post-stained with uranyl acetate, followed by Reynold's lead-citrate staining and then viewed on a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA).

4.4.5. Cellular fatty acid analysis

To determine whether chlorhexidine treatment influenced fatty acid composition of the cells, the following analysis was conducted. Biofilms were cultivated in the presence and absence of chlorhexidine (10 μg ml^{-1}) as described above. Biofilm cells were then scraped from the flow cell and collected in a 15 ml centrifuge tube followed by centrifugation at 4,000 rpm for 10 min. Total fatty acids were extracted from 40 to 50 mg (wet weight) of cell pellet and methyl-esterified as described by Annous *et al*. (1997). A Hewlett Packard 5890 series 2 gas-liquid chromatograph (Hewlett Packard, Avondale, PA) equipped with a flame ionization detector and a capillary column (Ultra 2; Hewlett Packard catalog no. 19091B-102; cross-linked 5% phenyl-methyl silicone; 25 m by 0.22 mm [inside diameter]; film thickness, 0.33 mm; phase ratio, 150) with hydrogen as the carrier gas was used for separation and detection of fatty acid methyl esters (FAMEs). The FAME peaks were automatically integrated by Hewlett Packard 3365 ChemStation software, and individual fatty acids were identified with the MIDI Microbial Identification Software (Sherlock TSBA Library version 3.80; Microbial ID, Inc., Newark, DE).

4.4.6. DIGE analysis of total cellular proteins

4.4.6.1. Experimental design, sample preparation and Cy-Dye Labeling

Control and chlorhexidine-treated MT51 and WT15 biofilms grown for 48 h under flowing conditions were aseptically recovered for extraction of protein, as previously described (Mangalapalli-Illathu *et al.,* 2008). In total, there were six biofilm treatment groups (WT15 control designated as WC, MT51 control (MC), WT15 treated at 10 μg ml^{-1} chlorhexidine (W10), WT15 treated at 30 μg ml^{-1} chlorhexidine (W30), MT51 treated at 10 μg ml^{-1} chlorhexidine (M10) and MT51 treated at 30 μg ml^{-1} chlorhexidine (M30)) with three biological replicates for each group, prepared independently from separate experiments. Sample preparation for DIGE analysis, which included cell lysis, protein extraction and solubilisation, was carried out as detailed previously (Sampathkumar *et al.,* 2004). The dye-binding assay of Bradford (1976) was then performed to quantify the extracted cellular proteins by using a protein assay kit (Bio-Rad Laboratories, Hercules, CA). The extracted protein was further precipitated using the 2D clean-up kit (GE Healthcare, Mississauga, ON) as per the supplier's protocol and resuspended in cell lysis buffer (7 M urea, 2 M thiourea, 4 % w/v CHAPS, 30 mM TrisCl, pH 9.0) to a final concentration of 5 μg/μl. The pH of the protein samples was adjusted to 8 and a total of 50 μg protein per sample was used for labelling reactions. The pooled internal standard was prepared by mixing equal amounts of protein from all treatments, and dispensing them in 50 μg aliquots prior to labeling.

Protein samples were labeled using fluorescent cyanine dyes (GE Healthcare) in accordance with the manufacturer's protocols. Cyanine dyes (Cy3 and Cy5) were freshly reconstituted in dimethylformamide and added to the labeling reactions at a ratio of 400 pmol dye to 50 μg protein. Labeling reactions were performed in the dark for 30 min and on ice, after which the reactions were terminated by the addition of 10 mM lysine (1 μl per 400 pmol dye). Each of the three replicates within a group was labeled with Cy3, Cy5 and Cy3 or Cy5. The pooled internal standard was labeled with Cy2 fluorescent dye. Sample multiplexing was also randomized to produce unbiased results.

4.4.6.2. Two-dimensional gel electrophoresis (2D-GE)

For every gel, 50 µg each of Cy3-labelled and Cy5-labelled protein samples were mixed with the Cy2-labelled pooled standard and added to rehydration buffer (7 M urea, 2 M thiourea, 2 % w/v CHAPS, 13 mM dithiothreitol (DTT), 0.5 % IPG buffer 4–7) to a total volume of 450 µl and loaded onto 24 cm, pH 4–7 IPG strips (GE Healthcare) and left overnight for rehydration. Isoelectric focusing (IEF) was performed using an Ettan IPGphor 3 (GE Healthcare) apparatus for a total of 57500 Vh at 75 µA and 20°C. The focused IPG strips were first equilibrated for 15 min with freshly added DTT (0.5% w/v) and then with iodoacetamide (4.5% w/v) for another 15 min in equilibration buffer (50 mM TrisCl (pH 8.8), 6 M urea, 30% glycerol, 2% w/v SDS and 0.02% bromophenol blue). Second-dimension electrophoresis was performed on 12.5% polyacrylamide gels using the Ettan DALT Six apparatus (GE Healthcare). Gels were run at 1 W per gel for 1 h, and at 17 W per gel at 20°C until the bromophenol blue tracking front had just run off the bottom of the gels.

4.4.6.3. Image acquisition and data analysis

Cy2, Cy3, and Cy5 images for each gel were scanned at 488/520-, 532/580-, and 633/670-nm excitation/emission wavelengths, respectively, at 100 µm resolution using a Typhoon FLA9000 scanner (GE Healthcare). The images were then cropped using ImageQuant v5.0 and processed using DeCyder v7.0 differential analysis software (GE Healthcare), as per the manufacturer's protocol. The differential in-gel analysis module of DeCyder software was used for spot co-detection and quantitation. The biological variation analysis (BVA) module was used for inter-gel matching of internal standard and samples across all gels and allowed quantitative comparisons of protein expression across all gels. ANOVA and Student's *t* tests were performed between different treatment groups. The Extended Data Analysis (EDA) module of the DeCyder software was also used for multivariate analysis of protein expression data after which Principal Component Analysis (PCA) was performed to find experimental outliers and patterns in expression data. DIGE data was exported and compared by principal

component analysis (PCA) with PRIMER v6 software. Statistical analyses of PCA scores generated from the first two component axes were run using an analysis of similarity (ANOSIM) with PRIMER v6 software (PrimerE-Ltd Lutton, UK). The following criteria was used to select proteins of interest: a) spots that showed a significant change in expression (P <0.01 and fold difference of more than 1.5, b) spots present in all gel images, and c) spots that gave a q score of more than 70 in cluster analysis. Finally, matches and data quality of proteins of interest were manually checked to avoid false positives. Forty-five spots were then marked as spots of interest to be picked for identification using mass spectrometry.

4.4.6.4. Protein identification

After second dimension electrophoresis, the pick gels were stained with Deep Purple fluorescent dye following the supplier's protocol (GE Healthcare). The gels were scanned in a Typhoon FLA9000 scanner at 100 µm resolution using the 532/LP 560 emission/excitation wavelengths. The preparative and analytical images were matched using the BVA module of the DeCyder software. Reference markers and spot picking locations were designated and edited as required. The pick list was then exported to the Ettan Spot picker (GE Healthcare) where the protein spots of interest were excised from the gel following the manufacturer`s protocol. The excised spot plugs were transferred to a 96-well microtitre plate, destained and digested using trypsin in the MassPrep II Proteomics Workstation (Micromass, UK) as per the method described by Sheoran *et al.* (2005). The proteins were then identified by liquid chromatography nano-electrospray ionization mass spectrometry (LC-ESI-MS). For LC-ESI-MS analysis, a Quadrupole Time-Of-Flight (Q-TOF) Global Ultima mass spectrometer (Micromass, Manchester, UK) equipped with a nano-electrospray (ESI) source and interfaced with a nanoACQUITY UPLC solvent delivery system (Waters, Milford, MA, USA) was used. The data generated from LC-ESI-MS was processed with the ProteinLynx Global Server 2.4 (PLGS 2.4, Waters) and subsequently submitted to Mascot (Matrix Science Ltd., London, UK) for peptide searching against the National Center for Biotechnology Information protein database. Gene ontology (GO) enrichment analysis was conducted using the Database for

Annotation, Visualization and Integrated Discovery (DAVID) to identify the biological processes and molecular functions of the proteins thus identified (Huang *et al.,* 2009).

4.5. Results

4.5.1. Identification of transposon insertion site and antimicrobial resistance patterns

To elucidate the molecular mechanism of chlorhexidine tolerance in *D. acidovorans*, we carried out transposon mutagenesis (Rema *et al.,* 2014) to create a chlorhexidine-susceptible mutant strain (MT51). The MIC of strain MT51 (1 μg ml^{-1}) for chlorhexidine was fifteen times lower than the parent strain WT15 (MIC-15 μg ml^{-1}). Identification of the DNA sequence flanking the transposon insertion site in this mutant revealed that the transposon was inserted into the 81^{st} bp of the 705-bp DNA region encoding the protein, TolQ. The sequence identity of this gene matched 100% to *D. acidovorans* SPH1 strain tolQ sequence published in the Nucleotide Database, National Center for Biotechnology Information (NCBI). Several attempts to create a complementation mutant to study the polar affects of *tolQ* mutation failed and consequently could not be examined in this thesis. Apart from chlorhexidine susceptibility, transposon mutation also rendered the MT51 strain less resistant to amikacin (MIC values of 16 and 64 μg ml^{-1} for MT51 and WT15, respectively) among the 17 antibiotics in the National Antimicrobial Resistance Monitoring System of the Centers for Disease Control panel (Table 4.1). MIC values for the other 16 antimicrobial agents in the screened panel were not observed to change appreciably following mutagenesis.

Table 4.1. Results of antibiotic and chlorhexidine susceptibility testing for *D. acidovorans* strains MT15 and WT51.

Antibiotic	MIC (µg ml^{-1})	
	Wild type	**Mutant**
Amikacin*	64	16
Chloramphenicol	16	16
Cefoxitin	<0.5	<0.5
Tetracycline	<4.0	<4.0
Ceftriaxone*	<0.25	0.5
Amoxycillin	1	1
Clavulanic acid	0.5	0.5
Ciprofloxacin*	0.25	0.12
Gentamicin	16	16
Nalidixic acid	0.5	0.5
Ceftiofur	0.5	0.5
Sulfisoxazole	32	32
Trimethoprim	0.12	0.12
Sulfamethoxazole	2.38	2.38
Kanamycin	64	64
Ampicillin	32	32
Streptomycin	64	64
Chlorhexidine*	15	1

MIC – minimum inhibitory concentration, * - indicates compounds for which differences in MIC were observed.

4.5.2. Transmission Electron Microscopy (TEM)

TEM was used to study the ultrastructural effects of chlorhexidine in both chlorhexidine-tolerant and chlorhexidine-susceptible *D. acidovorans* biofilm cells. Figures 4.1A and 4.1B are electron micrographs of cells recovered from 48 h untreated control WT15 and MT51 biofilms, respectively, and thus lack apparent damage to the cell envelop or coagulation of the cytoplasm. The WT15 biofilm cells treated at 10 μg ml^{-1} (Fig. 4.1C) appear similar to the WT15 untreated cells; however, the 10 μg ml^{-1} chlorhexidine treatment produced an effect on MT51, especially at the cell envelope where membrane bulging and detachment of cell membrane from the cell wall was observed (see arrows in Fig. 4.1D). Similar membrane waviness and damage was apparent in both MT51 and WT15 biofilms treated with 30 μg ml^{-1} chlorhexidine (Fig. 4.1F), suggesting that a lethal dose of chlorhexidine exerts membrane-level effects on *D. acidovorans* cells.

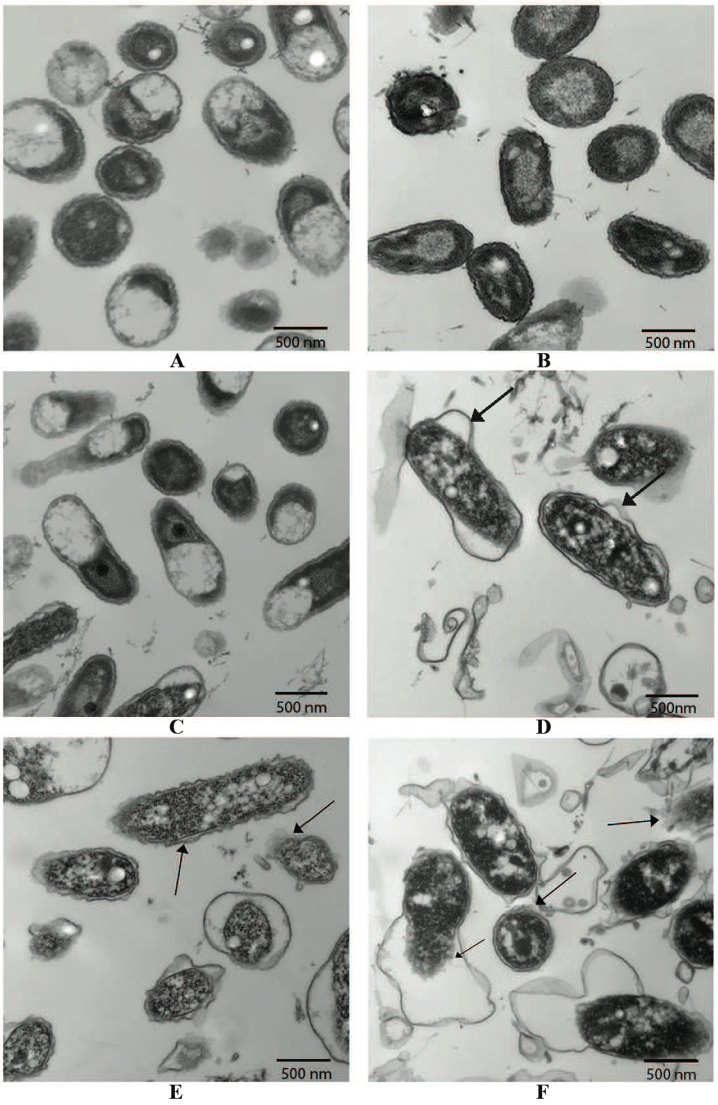

Fig. 4.1. TEM micrographs of 48 h *D. acidovorans* biofilm cells with and without exposure to 10 and 30 μg ml^{-1} chlorhexidine after 24 h of growth. A-WT15 Control; B-MT51 Control; C-WT15 treated with chlorhexidine at 10 μg ml^{-1}; D-MT51 treated with chlorhexidine at 10 μg ml^{-1}; E-WT15 treated with chlorhexidine at 30 μg ml^{-1}; F-MT51 treated with chlorhexidine at 30 μg ml^{-1}. Arrowheads show membrane structural alterations. Scale bar = 500 nm.

4.5.3. Cellular fatty acid analysis

Treated (10 μg ml^{-1} chlorhexidine) and untreated biofilms of chlorhexidine-tolerant WT15 and chlorhexidine-susceptible MT51 biofilms were subjected to fatty acid methyl ester (FAME) analysis. The fatty acids detected in WT15 control biofilms after 48 h of growth (Fig. 4.2) included both saturated fatty acids (12:0 and 16:0) and unsaturated fatty acids (16:1ω7c/15 iso2OH, 17:1ω5c, and 18:1ω7c). Treatment of chlorhexidine-tolerant WT15 biofilms with 10 μg ml^{-1} chlorhexidine resulted in a reduction in the concentration of total unsaturated fatty acids from an initial value of 68.2% to 63.6% (Fig. 4.2), apparently due to the disappearance of 17:1ω5c fatty acid. Simultaneously, there was a minor increase in saturated fatty acids and the appearance of a new group, the cyclic fatty acids (17:0 cyclo), in the chlorhexidine-treated wild type biofilms. The unsaturated fatty acid, 17:1ω5c, was not found in mutant MT51 biofilms. When MT51 biofilms were exposed to chlorhexidine (10 μg ml^{-1}), the level of unsaturated fatty acids increased slightly from 68.4% to 70.1%, and no production of cyclic fatty acids was observed.

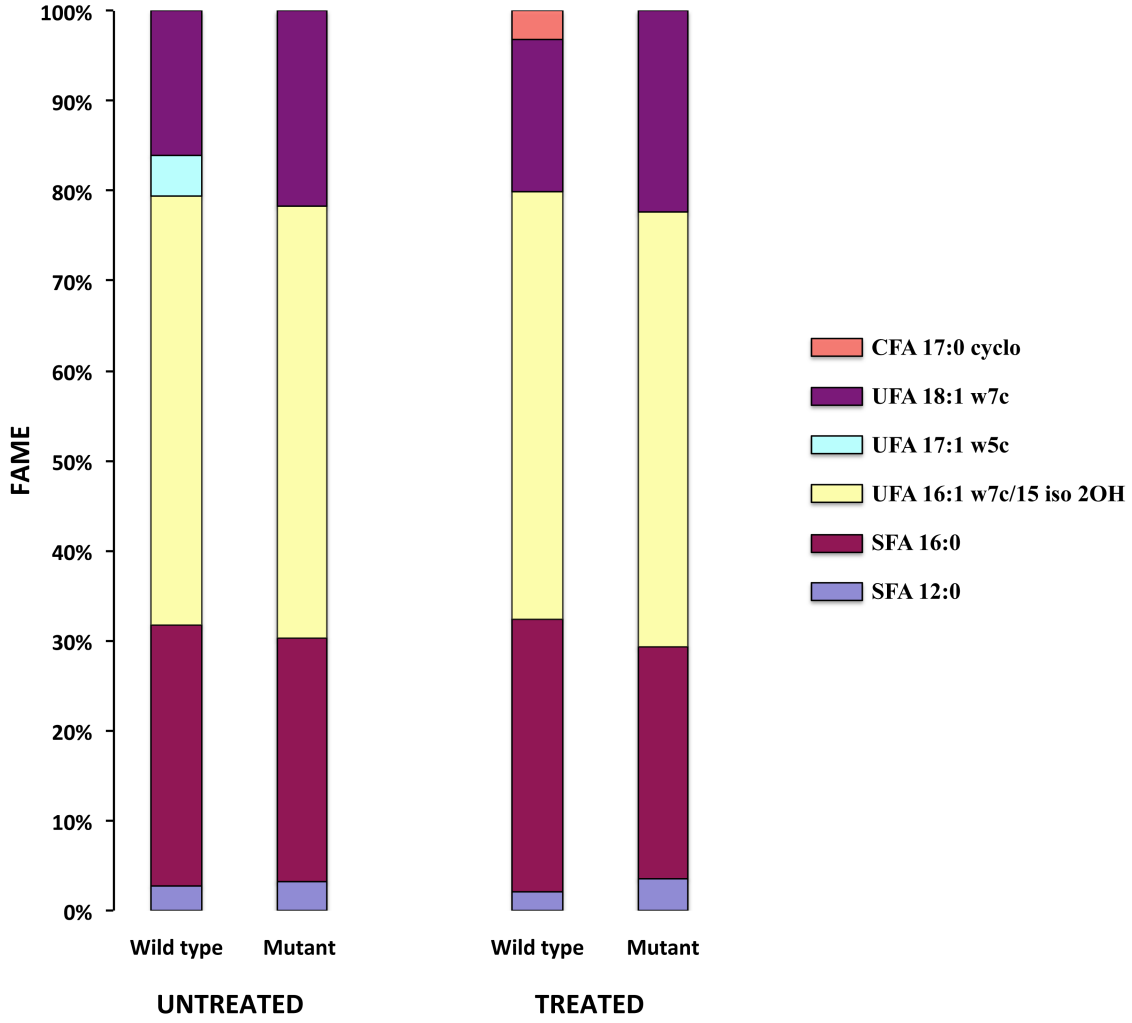

Fig. 4.2. Relative amounts (%) of fatty acid methyl esters, determined using gas chromatography, found in chlorhexidine-tolerant (WT15) and chlorhexidine-susceptible (MT51) *D. acidovorans* biofilm cells treated with (10 μg ml^{-1} with chlorhexidine) compared with the untreated control. CFA- cyclo fatty acids, UFA – unsaturated fatty acids, SFA – saturated fatty acids.

4.5.4. Proteomic analyses

DIGE analyses were carried out using: 1) 48 h WT15 and MT51 control biofilms receiving no chlorhexidine, and 2) WT15 and MT51 biofilms treated for 24 h with 10 and 30 μg ml^{-1} chlorhexidine after 24 h of initial biofilm growth. Principal Component Analysis (PCA) was used to group the 18 individual Cy3- or Cy5-labeled spot maps based on the overall expression pattern of all 999 spots matched between gels, and to identify any experimental outliers. The PCA of spot maps (Fig. 4.3) demonstrated a high reproducibility between replicate samples, as indicated by close grouping of the experimental replicates. The first principal component accounted for 43.7% of total variance and clearly separated the MT51 control, MT51 treated (10 μg ml^{-1}) and WT15 control groups from each other and indicated little separation between the WT15 control and WT15 treated biofilms. However, the second principal component, which accounted for 17% of the variance, differentiated WT15-treated groups from the other experimental groups. PCA results indicated significant differences between mutant and wild type control biofilms (ANOSIM, $p<0.001$). Mutant treated biofilms tended to group together (ANOSIM, $p<0.001$) indicating similar proteomic response. Overall, PCA demonstrated that both strains were differentially-affected by chlorhexidine treatment relative to their untreated controls.

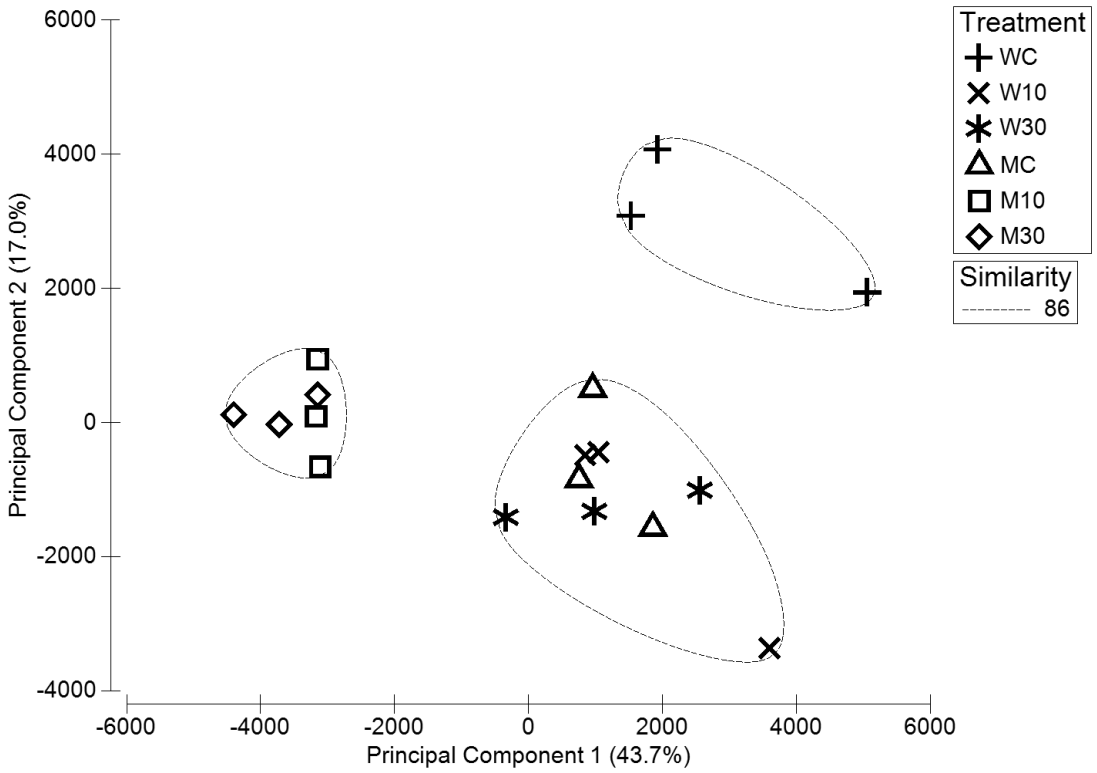

Fig. 4.3. Principal Component Analysis of the 18 individual DIGE expression spot maps (6 treatments with three replications per treatment) differentiated by principle components one and two. Statistical analyses of PCA scores generated from the first two component axes were run using an analysis of similarity (ANOSIM) with PRIMER v6 software. M10, M30 - mutant strain treated with 10 and 30 µg ml^{-1} chlorhexidine, respectively; W10 and W30 – wild type strain treated with 10 and 30 µg ml^{-1} chlorhexidine, respectively; MC and WC –mutant and wild type controls respectively.

DIGE analysis revealed numerous protein species-abundance changes in both wild type and mutant biofilms when exposed to sub-MIC (10 μg ml^{-1}) and above-MIC (30 μg ml^{-1}) chlorhexidine levels. The number of differentially expressed proteins among various groups compared is shown in Table 4.2. For example, 114 proteins were differentially expressed between untreated control WT15 and MT51 biofilms, of which 70 were up regulated and 44 were down-regulated in MT51. Table 4.2 also shows that the number of differentially-expressed proteins with respect to the two chlorhexidine concentrations was 9 (5 were up-regulated and 4 were down-regulated) in WT15 biofilms; whereas, 56 proteins (30 were up-regulated and 26 were down-regulated) were differentially-expressed in MT51 biofilms. This finding is in keeping with our TEM data (Fig. 4.1) that showed that MT51 was more dramatically affected by chlorhexidine treatment. Two-way ANOVA analysis showed that treatment-specific differential expression of proteins was more profound than the effect of the *tolQ* mutation ($P< 0.05$), as 106 proteins were differentially-expressed (among the mutant and wild type) across all treatments; whereas, only 60 proteins were affected due to transposon insertion. A further breakdown of the differential protein abundance according to fold-change, is shown in Figure 4.4. Chlorhexidine treatment induced higher fold-changes (>1.5) in both WT15 and MT51 biofilms compared to differences in protein expression between the respective untreated controls. However, the protein expression changes in the same biofilm treated with 30 μg ml^{-1} chlorhexidine were comparatively higher in MT51 than the WT15 biofilms relative to their respective biofilms exposed to 10 μg ml^{-1}. Only one spot each showed increased and decreased abundance of 2- to 3-fold in wild type biofilms treated with high versus low concentrations of chlorhexidine;

Table 4.2. Comparison data of differentially expressed proteins (fold increase/decrease ≥ 1.5, P <0.05) among various experimental groups.

Groups compared	Proteins differentially expressed	Proteins picked for identification	Increased abundance	Decreased abundance	Highest fold increase	Highest fold decrease
MC/WC	114	25	70	44	6.28	15.19
W10/WC	105	26	53	52	6.25	7.54
W30/WC	83	27	49	34	14.87	7.49
M10/MC	107	30	63	44	7.62	4.47
M30/MC	119	39	69	50	7.0	7.14
M10/W10	138	40	92	46	5.4	5.75
M30/W30	111	30	39	72	4.43	8.26
W30/W10	9	4	5	4	2.36	2.77
M30/M10	56	11	30	26	2.25	3.62
2-WAY ANOVA (strain)	60	13	NA	NA	NA	NA
2-WAY ANOVA	106	31	NA	NA	NA	NA
2-WAY ANOVA	54	14	NA	NA	NA	NA

NA indicates not applicable

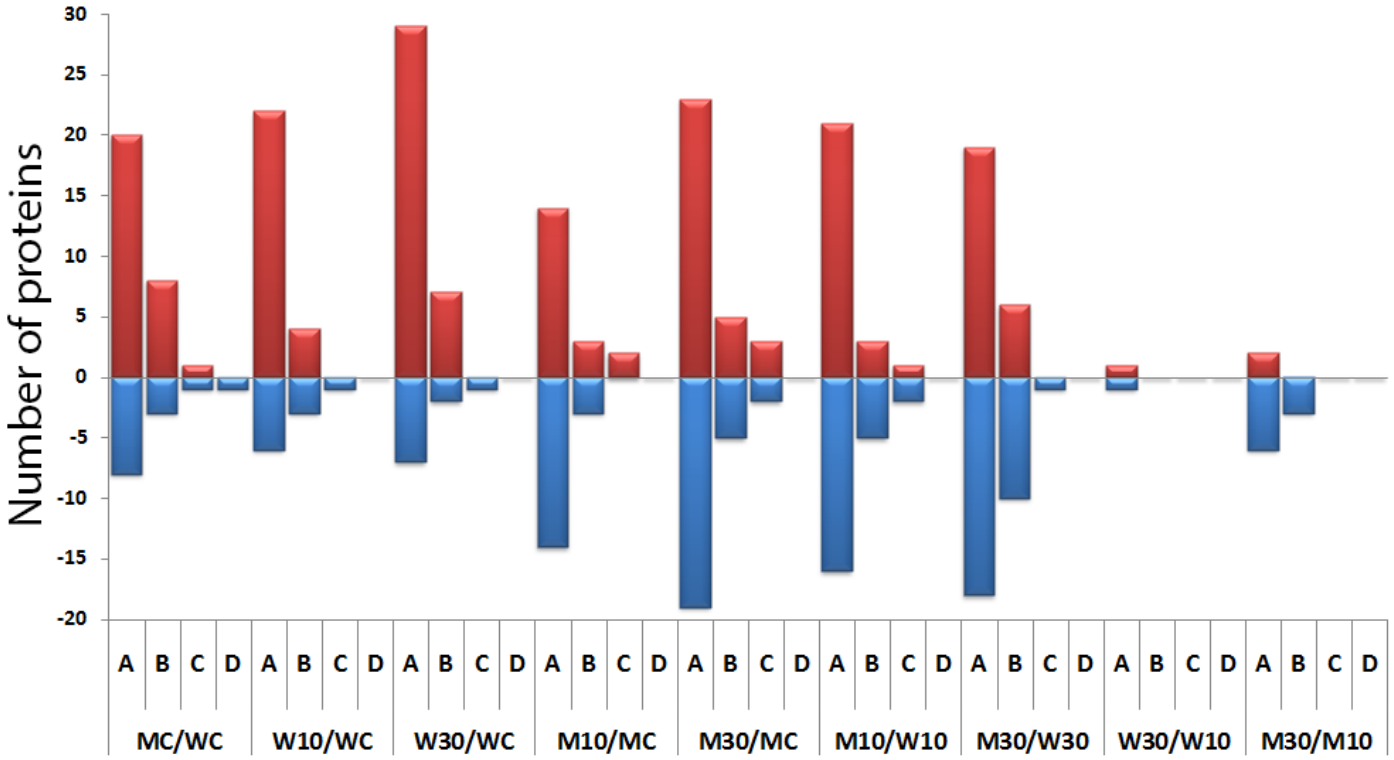

Fig. 4.4. Number of up-regulated and down-regulated proteins among various levels of significant fold change between various experimental grou Red bars indicate the number of up-regulated proteins and the blue bars show the number of down-regulated proteins. A, B, C, D stands for 2-3, 3-5, 10 and above 10-fold changes, respectively. $P< 0.05$. M10, M30 - mutant strain treated with 10 and 30 µg ml^{-1} chlorhexidine, respectively; W10 a W30 – wild type strain treated with 10 and 30 µg ml^{-1} chlorhexidine, respectively; MC and WC –mutant and wild type controls respectively.

whereas, 2 spots with increased, and 7 spots with decreased, abundance of 2- to 3-fold were seen in mutant biofilms.

Out of 45 differentially-expressed proteins picked, 40 proteins were successfully identified and are summarized in Table 4.3, along with their molecular functions, accession numbers and abundance fold-changes. Based on their known functions and Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis, these identified proteins were broadly categorised as those involved in amino acid and lipid biosynthesis, translation/transcription, energy metabolism, membrane and stress-related functions. DAVID analysis (Fig. 4.5) shows the major biological and molecular processes that were affected due to chlorhexidine treatment in WT15 and MT51 biofilms. The most pronounced changes observed were the down-regulation of proteins involved in biological processes, as listed in Figure 4.5. Of the forty identified proteins, 12 proteins increased in abundance in both strains (WT15 and MT51) at both concentrations of chlorhexidine. These included chaperonin GroEL, aspartyl/glutamyl-tRNA amidotransferase subunit A, F0F1 ATP synthase subunit alpha, elongation factors Tu and Ts, phosphopyruvate hydratase, amidohydrolase, basic membrane lipoprotein, electron transfer flavoprotein and ATP-dependent Clp protease. Those that were decreased in abundance in both WT15 and MT51 biofilms included ribosomal subunit interface protein, AMP-binding domain-containing protein and a hypothetical protein (ferritin-like superfamily). The chaperonin protein, ClpP (spot no. 658, Table 4.3), was found to be up-regulated in WT15 biofilms exposed to the inhibitory (30 μg ml^{-1}) chlorhexidine concentration and was higher and almost similar in both 10 μg ml^{-1} and 30 μg ml^{-1} treated MT51 biofilms, suggesting that both these

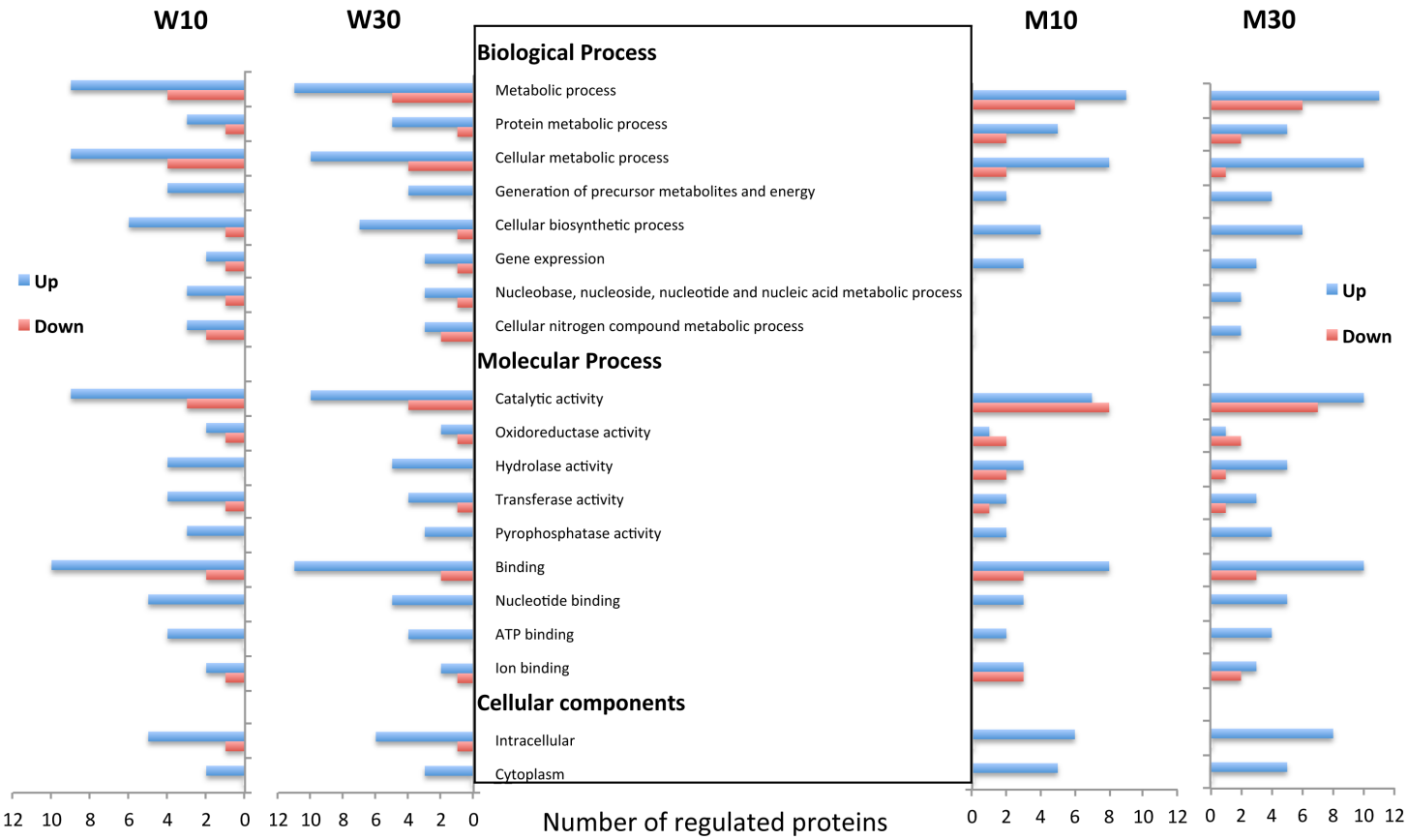

Fig. 4.5. Gene ontology cluster (DAVID) analysis of proteins up-regulated (blue) and down-regulated (red) in WT15 and MT51 biofilms treated w chlorhexidine at 10 and 30 μg ml^{-1} showing the biological, molecular and cellular processes that were affected. The proteins were picked from the de purple-stained pick gel and analyzed by mass spectrometry.

chlorhexidine concentrations were stressful to MT51. Similarly, the chaperonin, GroEL, was also significantly up regulated in MT51 chlorhexidine-treated biofilms compared with WT15 chlorhexidine-treated biofilms. Several protein spots with slightly different electrophoretic migration patterns were determined to be the same protein (i.e., chaperonin GroEL, ATP synthase and elongation factor Tu), which may imply that charge or post-translational modifications had occurred (dos Santos *et al.,* 2010). Another protein, the small heat shock protein HSP 20 (spot no. 886, Table 4.3), which is an ATP-independent molecular chaperone, was also significantly down-regulated by nearly 7-fold only in wild type chlorhexidine-treated biofilms. There was also a more than 2-fold decrease in expression of phasin proteins (Table 4.3) in WT15 chlorhexidine-exposed biofilms compared to MT51 biofilms. Oxidative stress proteins, namely superoxide dismutase, hypothetical protein (alkylhydroperoxidase-like protein, AhpD family) and redoxin domain-containing protein, were found to be either down-regulated or showed no significant changes in WT15 and MT51 biofilms (Table 4.3).

Consistent with our FAME results, several enzymes associated with fatty acid synthesis were also found to be affected by chlorhexidine treatment in both WT15 and MT51 biofilms. They included malonyl CoA-acyl carrier protein transacylase (spot no. 478), enoyl-CoA hydratase/isomerase (spot no. 563) and (3R)-hydroxymyristoyl-ACP dehydratase (spot no. 770). A dramatic increase, from ~3.3- to 7.5-fold, in the expression of a basic membrane lipoprotein (spot no. 371, Table 4.3) in both WT15 and MT51 chlorhexidine-treated biofilms indicated that this protein may play a very important role in chlorhexidine tolerance in *D. acidovorans*. A role for quorum sensing is also suggested by the down-regulation of the two-component LuxR family transcriptional regulator (spot no. 629, Table 4.3), especially in WT15 biofilms treated with the high concentration of chlorhexidine.

Table 4.3. Protein expression changes in 48 h *D. acidovorans* WT15 and MT51 biofilms following CHX treatments at 10 (W10, M10) and (W30, M30) μg ml^{-1} after 24 h of biofilm growth.

Spot No.	Proteins and associated Biological process	Accession No.	Mass	pI	Fold change[b]							2-way ANOV
					MC/WC	W10*	W30*	M10*	M30*	M10/W10	M30/W30	
	Generation of precursor metabolites and energy (GO:0006091)[a]											
115	ATP synthase subunit alpha	gi|160895867	55205	5.64	+4.07	+2.76	+2.91	NS	NS	NS	+2.16	S,T,I
122	ATP synthase subunit alpha	gi|160895867	55205	5.64	+2.81	+1.90	+2.45	NS	+1.81	+2.07	+2.07	S,T,I
125	ATP synthase subunit beta	gi|160895869	50633	5.23	+2.68	+2.22	+3.08	+1.64	+2.84	+1.98	+2.48	S,T,I
995	ATP synthase subunit beta	gi|160895869	50633	5.23	+2.01	+2.12	+2.24	+1.88	+1.75	+1.79	NS	S,T
589	Ubiquinol-cytochrome c reductase iron-sulfur subunit	gi|160900935	21695	6.1	NS	-1.84	-2.80	NS	NS	NS	NS	T,I
200	Enolase	gi|160900540	45951	4.82	+2.24	+2.92	+3.27	+2.98	+3.54	+2.29	+2.43	S,T
	Fatty acid metabolic process (GO:0006631)											
478	Malonyl CoA-acyl carrier protein transacylase	gi|160900703	34368	5.32	+2.21	+1.35	+1.82	NS	NS	NS	NS	S,T
770	(3R)-hydroxymyristoyl-ACP dehydratase	gi|160900369	16579	5.92	NS	NS	NS	+1.72	+2.22	NS	NS	S,T,I
563	Enoyl-CoA hydratase/isomerase	gi|160896976	27994	5.39	+2.50	NS	NS	-2.63	-3.82	NS	NS	T,I
371	Basic membrane lipoprotein	gi|160897585	41068	5.71	NS	+3.30	+6.33	+7.62	+6.20	NS	NS	S,T,I
	Nucleotide biosynthetic process (GO:0009165)											
606	Glutamine amidotransferase	gi|160899150	25022	5.72	-15.19	-3.63	-3.42	NS	NS	-5.09	-3.46	S,T,I
218	Amidohydrolase	gi|160897029	45940	5.03	NS	+2.11	+2.43	+1.92	+2.16	NS	NS	S,T
91	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	gi|160897011	57189	5.72	+2.26	+1.79	+2.18	+1.7	NS	NS	NS	S,T
	Molecular chaperones / protein folding (GO:0006457)											
877	FKBP-type peptidylprolyl isomerase	gi|160900492	12314	5.23	-2.02	-1.73	-1.53	NS	NS	-1.59	-1.83	S,T
544	PpiC-type peptidyl-prolyl cis-trans isomerase	gi|160898739	28914	8.5	-1.69	NS	NS	-1.50	-2.33	-1.93	-2.58	S,T
886	Heat shock protein HSP20	gi|160897822	13608	5.78	-9.43	-7.54	-7.49	NS	NS			S,T,I
59	60 kDa chaperonin	gi|160901092	57123	5.02	NS	+1.85	+3.36	+3.35	+7.0	+1.59	+1.84	S,T,I
85	60 kDa chaperonin	gi|160901092	57123	5.02	NS	NS	NS	+2.02	+2.92	+2.05	+2.14	S,T,I
86	60 kDa chaperonin	gi|160901092	57123	5.02	NS	NS	NS	+2.03	+2.49	+1.77	+1.89	S,T,I
	Translation/Transcription (GO:0006412/0006351)											
95	Aspartyl/glutamyl-tRNA amidotransferase subunit A	gi|160895801	52727	5.51	+2.30	+2.47	+2.65	+2.19	+2.35	+2.04	NS	S,T
190	Elongation factor Tu	gi|160895838	43296	5.48	NS	+2.06	+2.38	+2.53	+3.25	NS	+2.42	S,T,I
274	Elongation factor Tu	gi|160895838	43296	5.48	NS	+3.81	+4.31	NS	+2.01	NS	NS	T,I
436	Elongation factor Ts	gi|160900379	31142	5.5	NS	NS	+2.07	+1.76	+2.06	NS	NS	T
629	Two component LuxR family transcriptional regulator	gi|160899416	23896	5.87	NS	-1.64	-1.97	NS	NS	NS	NS	S,T,I

Spot No.	Proteins and associated Biological process	Accession No.	Mass	pI	Fold change[b]							2-way ANOV
					MC/WC	W10*	W30*	M10*	M30*	M10/W10	M30/W30	
	Electron carrier activity (GO:0009055)											
292	Taurine catabolism dioxygenase TauD/TfdA	gi|160900189	37395	5.17	NS	+2.17	+2.49	NS	NS	-1.83	NS	S,T,I
612	Electron transfer flavoprotein subunit alpha/beta	gi|160896849	26734	7.64	NS	NS	+1.66	+1.96	+3.08	+1.57	+1.67	S,T,I
	Oxygen and reactive oxygen species metabolic process (GO:0006800)											
662	Superoxide dismutase	gi|160899011	21614	5.87	NS	-3.76	-4.56	NS	NS	NS	+3.39	S,T,I
647	Superoxide dismutase	gi|160898411	22797	5.86	-2.19	NS	NS	NS	NS	NS	-3.62	
644	Hypothetical protein (alkylhydroperoxidase like protein, AhpD family)	gi|160899719	23967	5.87	NS	NS	NS	-3.03	NS	-2.92	NS	S,T,I
	Proteolysis (GO:0006508)											
658	ATP-dependent Clp protease proteolytic subunit	gi|160898086	22274	5.45	NS	NS	+1.49	+1.77	+1.87	NS	NS	S,T,I
75	Hypothetical protein (peptidase dimerisation domain protein)	gi|160896656	62743	7.08	NS	NS	NS	-2.66	-2.48	-2.58	-2.51	S,T,I
	Antibiotic metabolic process (GO:0016999)											
536	Hydroxyacylglutathione hydrolase	gi|160900329	28066	5.34	+6.28	NS	NS	-2.59	NS	+3.77	+4.23	S,T
	Phosphorus metabolic process (GO:0006793)											
687	Inorganic diphosphatase	gi|160900311	19368	4.96	NS	NS	NS	+1.43	+2.02	NS	NS	T,I
	Cell redox homeostasis											
734	Redoxin domain-containing protein	gi|160895850	20383	5.63	NS	NS	NS	NS	-2.78	NS	NS	S,T,I
	Transaminase activity (GO:0008483)											
319	Class V aminotransferase	gi|160896522	41574	6.08	NS	NS	NS	NS	-6.12	-2.05	-4.33	S,T,I
	Ion bonding (GO:0043167)											
416	Hypothetical protein (ferritin-like superfamily of di-iron-containing four helix-bundle protein)	gi|160901296	34343	4.86	+3.97	NS	-1.55	-2.45	-2.71	+2.23	+2.26	S,T
	Others/Unknown											
595	Glutathione S-transferase domain-containing protein	gi|160896300	23088	6.17	NS	NS	NS	NS	+2.41	+1.73	NS	S,T,I
808	Ribosomal subunit interface protein	gi|160896367	13610	5.51	-1.96	-1.73	NS	-1.66	-1.96	-1.87	-2.64	S,T
46	AMP-binding domain-containing protein	gi|160901406	63817	5.63	NS	NS	-1.62	-4.47	-7.14	-3.13	-2.98	S,T,I
714	Phasin family protein	gi|160900174	19110	5.43	-2.35	-2.14	-2.11	NS	NS	NS	NS	S,T,I

*Fold change in protein expression from their respective control biofilms. + indicates increased expression, - indicates decreased expression, GO – Gene Ontology ID. NS- not significant, S- strain (WT15 and MT51), T-treatment (0, 10 and 30 μg ml^{-1}), I- interaction.

Chlorhexidine treatment also doubled the abundance of amidohydrolase enzyme in both WT15 and MT51 biofilms (spot no. 218, Table 4.3). Similarly, proteins involved in protein synthesis, folding and stabilisation (GroEL, ClpP protease, aspartyl/glutamyl-tRNA amidotransferase, elongation factors Tu and Ts) and a few proteins associated with energy production, nucleotide transport and metabolism increased in abundance (i.e., bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase, phosphopyruvate hydratase, F0F1 ATP synthase subunit alpha/beta and electron transfer flavoprotein). However, the enzyme glutamine amidotransferase (spot no. 606, Table 4.3), also involved in nucleotide transport and metabolism, decreased in abundance by almost 3.5 times in WT15 biofilms at both chlorhexidine concentrations; whereas, the taurine catabolism dioxygenase TauD/TfdA (spot no. 292) abundance increased more than two-fold in WT15 biofilms treated with chlorhexidine.

The major differences between the wild type and mutant control biofilms were the increase in abundance of the ATP synthases, a few enzymes involved in fatty acid metabolism, and hydroxyacylglutathione hydrolase and decrease in abundance of glutamine amidotransferase, several molecular chaperones and family phasin protein in mutant biofilms compared to their parent biofilms (Table 4.3).

4.6. Discussion

Mutation of *tol* genes has previously been shown to confer hypersensitivity to various agents, such as detergents, quaternary compounds, and some antibiotics in *Escherichia coli* (Dennis *et al.,* 1996). However, little is known about TolQ's functional role in *D. acidovorans.* In *E. coli*, TolQ is an integral cytoplasmic membrane protein (230 amino acids) with three membrane-spanning regions required for maintenance of the integrity of the bacterial envelope (Vianney *et al.,* 1994). The *tolQRAB-pal* operon also functions in the uptake of certain bacteriophages and colicins and is conserved in Gram negative genomes (Dennis *et al.,* 1996). Studies also suggest that the Tol/Pal system might anchor the OM to the peptidoglycan layer (Clavel *et al.,* 1998) and further may

catalyse porin biogenesis or activity (Dover *et al.,* 2000). It was thus hypothesised that a mutation in *tolQ* in *D. acidovorans* would affect the cell envelope's integrity upon exposure to chlorhexidine, a membrane-active agent (Hope and Wilson, 2004). Due to the absence of complete knowledge of TolQ in *D. acidovorans*, along with limitations in the present study and failed attempts for complementation studies, it is possible that TolQ influenced the activity of other proteins not detected in our DIGE analysis. For example, Omp32, undetected in our analyses, has been found to have strong anion selectivity and can repulse the penetration of positively-charged compounds such as chlorhexidine (Zeth *et al.,* 2000). Previously, we demonstrated that more chlorhexidine was found to accumulate inside chlorhexidine-susceptible MT51 cells than in chlorhexidine-tolerant cells (Rema *et al.,* 2014), clearly pointing to selectivity in chlorhexidine penetration.

TEM has seen extensive use to study the effects of antimicrobial agents on microorganisms (Sampathkumar *et al.,* 2004; Vitkov *et al.,* 2005). Our TEM analyses provided clear evidence that chlorhexidine has a disruptive effect on the *D. acidovorans* cell envelope, in agreement with previous reports for other bacteria (Richards and Cavill, 1979; Russell, 2003; Gilbert and Moore, 2005). These observations are also consistent with the *tolQ* mutation influencing membrane integrity of *D. acidovorans* cells, wherein the mutant cells appeared more susceptible to chlorhexidine than the wild type (Fig. 4.1). Tattawasart *et al.* (Tattawasart *et al.,*2000) observed that chlorhexidine-resistant *Pseudomonas stutzeri* cells were larger, had fibril-like structures on their outer surfaces, and the cell envelope was wavier and thicker than those of susceptible isolates. Time-dependent cellular damage (i.e., blebbing of the outer membrane, cytoplasmic swelling) was also been seen in the case of susceptible isolates when treated with chlorhexidine (Tattawasart *et al.,* 2000). These authors also observed extensive cell lysis, likely the consequence of exposing the cells to a significantly-higher chlorhexidine concentration (100 μg ml^{-1}) than employed in our study. The type of bacteria, planktonic versus biofilm, chlorhexidine concentration, and period of exposure all play important roles in inducing ultrastructural changes in microorganisms (Vitkov *et al.,* 2005).

Changes in cell fatty acid composition in the presence of chlorhexidine possibly reflect alterations in membrane fluidity and symmetry, and offer a potential mechanism of chlorhexidine tolerance in *D. acidovorans*.

There were some differences in the major fatty acids detected in our chlorhexidine-tolerant *D. acidovorans* (Fig. 4.2) after 48 h treatment compared with the *D. acidovorans* of Wen *et al.* (1999). These differences were likely due to the combined effect of using biofilm (our study) versus planktonic cells, as well as differences in growth conditions. In our study, chlorhexidine treatment caused a reduction in total unsaturated fatty acids associated with the disappearance of the 17:1ω5c fatty acid, and the *de novo* appearance of 17:0 cyclo fatty acids in the wild type strain. Such a change in membrane fatty acid composition has been associated with decreased membrane fluidity (Annous *et al.,* 1997; Brown *et al.,* 1997). Abu-Elteen and Whittaker (1997) observed that the percentage of unsaturated fatty acids decreased while the ratios of C16:C18 increased in the presence of chlorhexidine in *Candida albicans*. Chlorhexidine has also been observed to alter the cell-membrane lipid packing order and membrane fluidity in human epithelial cells (Audus *et al.,* 1992). The fatty acid compositional modifications (Fig. 4.2) that were seen in WT15 biofilms in response to chlorhexidine treatment were not evident in the mutant chlorhexidine-treated biofilm, and thus appear to be part of the *D. acidovorans* adaptive response.

DIGE has previously been used to elucidate the proteomes of bacterial drug resistance (dos Santos *et al.,* 2010; Fernandez-Reyes *et al.,*2009; Poutanen *et al.,* 2009). Our study is the first to investigate chlorhexidine-induced changes in the *D. acidovorans* proteome. PCA analysis of the DIGE gels (Fig. 4.3) indicated that the effect of chlorhexidine on MT51 biofilm protein expression was distinct from its effect on the WT15 biofilms, and that both chlorhexidine-treated biofilms were different from their respective control biofilm conditions. These differences appear due to the mutation in the *tolQ* gene in MT51, as TolQ has previously been shown to influence antimicrobial resistance and membrane integrity in other bacterial cells (Dennis *et al.,* 1996; Vianney *et al.,* 1994). PCA analyses also confirmed significant differences (ANOSIM, $p<0.001$) between WT15 and MT51 control biofilms due to *tolQ* mutation. Since WT15 is chlorhexidine-tolerant (Rema *et al.,* 2014) (Table 4.1), we did not expect a large difference between the control and chlorhexidine-treated proteomes, especially at the lower concentration. In contrast, both low and high chlorhexidine concentrations used in this study were above the MIC determined for MT51 cells and so MT51 treated biofilms were significantly different from MT51 control biofilms (ANOSIM, $p<0.001$). Hence, it was hypothesized that the MT51 biofilms would experience greater chlorhexidine-

induced stress than the WT15 biofilms, and this would be reflected by their proteome response. Previously, microscopic analyses (Rema *et al.,* 2014) also showed greater effects of chlorhexidine on MT51 biofilms. Accordingly, the first principal component (PC1) showed that a greater differential effect on protein expression did in fact result from application of the two chlorhexidine concentrations (10 and 30 μg ml^{-1}) on MT51 biofilms.

Bacterial stress response is a coordinated outcome of the expression of a variety of genes that alter various cellular processes (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.) and the number of genes involved are numerous (Renzone *et al.,* 2005). Information on the proteome of bacterial species exposed to chlorhexidine, or those of chlorhexidine-resistant isolates, are limited. Recently, Coeyne *et al.* (2011) used microarray analysis to assess molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms, revealing that 15 min exposure of *B. cenocepacia* to 0.15 μg ml^{-1} chlorhexidine resulted in the up-regulation of 469 (6.56% of the total) and down-regulation of 257 (3.59% of the total) protein-coding genes (fold-change > 2; $P < 0.05$). Similarly, the analysis of DIGE gels in our study revealed numerous protein species-abundance changes in both wild type and mutant biofilms when exposed to sub- and above-MIC levels of chlorhexidine (Table 4.2). A greater number of proteins were affected (Fig. 4.4) in chlorhexidine-treated mutant versus untreated control biofilms than the treated and untreated wild type biofilms, suggesting that the MT51 mutant was more dramatically-affected by chlorhexidine exposure, in keeping with our TEM and other observations. Two-way ANOVA ($P < 0.05$) analysis (Table 4.3) showed that treatment-specific differential expression was profound when compared to strain, as the proteome response in MT51 cells was the integrated effect of the mutated genetic background as well as above-MIC chlorhexidine exposure.

A wide range of key enzymes and proteins involved in various biological and molecular processes were affected by chlorhexidine, as shown by gene ontology analysis (Fig. 4.5). The DIGE proteomic data revealed that the differentially-expressed proteins in these various treatment groups fell into several functional groups, mainly those involved in energy, nucleotide, fatty acid and amino acid metabolism, protein translation and modification, DNA binding and transcription, cell membrane-related functions and other cellular processes such as detoxification

and stress response (Table 4.3). The cytoplasmic membrane would be the highest-probability target for biocide action for chlorhexidine, causing functional perturbation of the membrane (Denyer and Stewart, 1998). This implies that the target enzymes and proteins for biocide action would be mostly those related to structural integrity, transport mechanisms, energy coupling processes, respiratory chain function and those that are membrane-bound (Denyer and Stewart, 1998), and this was consistent with our observations (Table 4.3). The majority of *B. cenocepacia* biofilm protein-coding genes over-expressed when treated with chlorhexidine coded for periplasmic and exporter proteins or lipoproteins, efflux systems and those that were required for structure and function of the inner membrane, transport or binding, chemotaxis and motility (Coenye *et al.,* 2011). The majority of down-regulated protein-coding genes were involved in transport or binding, or regulatory functions. Similarly, treatment of *Pseudomonas aeruginosa* planktonic cells with 0.008 mM chlorhexidine for 10 and 60 min revealed that membrane transport, oxidative phosphorylation, and electron transport genes were down-regulated (Nde *et al.,* 2009). The *opr*H gene and MexCD-OprJ multidrug efflux pump were among the up-regulated proteins found to play a major role in chlorhexidine resistance in *P. aeruginosa* (Nde *et al.,* 2009).

In our study, several stress response-related proteins were found to be differentially-expressed in the presence of chlorhexidine, including GroEL and ATP synthase. Other authors have made similar observations in organisms under antimicrobial stress (Poutanen *et al.,* 2009; Cardoso *et al.,* 2010; Karatzas *et al.,* 2008). Overexpression of GroEL in MT51 biofilms may be directly related to the sensitivity of this strain to chlorhexidine and their stressed metabolic state. It further indicates that there may be an increased concentration of misfolded proteins in MT51 biofilms and chlorhexidine may interfere with protein synthesis or structure (Cardoso *et al.,* 2010). Similarly, there is evidence for the role of housekeeping genes, such as glutamyl-tRNA amidotransferase and elongation factors Tu and Ts, in biocide tolerance (Sampathkumar *et al.,* 2004). Clp protease is a periplasmic chaperone protein that plays a major role for bacterial survival under stress conditions where proteins tend to unfold and aggregate (dos Santos *et al.,* 2010; Frees *et al.,* 2004). ClpP was found to be up-regulated in both WT15 and MT51 upon chlorhexidine treatment, though the induction level of ClpP was higher at both concentrations in MT15 biofilms. In *Staphylococcus aureus*, Clp ATPases are required for stress tolerance against a broad range of

inducing factors (Frees *et al.,* 2004). Induction of this protease upon chlorhexidine treatment indicates an effect on periplasmic proteins in order to decrease the toxic effect of misfolded proteins. However, the small heat shock protein, HSP 20, which is an ATP-independent molecular chaperone, was down-regulated by nearly 7-fold in WT15 chlorhexidine-treated biofilms, indicating this protein did not play a significant role in chlorhexidine tolerance (Vetura *et al.,* 2007). Superoxide dismutase was also down-regulated in WT15 biofilms or not changed significantly in mutant biofilms, suggesting the absence of oxidative stress during chlorhexidine exposure. Other studies have made similar findings using different antimicrobial agents (dos Santos *et al.,* 2010). The lack of oxidative stress is further substantiated by the down-regulation or absence of change in the hypothetical protein (alkylhydroperoxidase-like protein, AhpD family) and redoxin domain-containing protein that may have a role in attenuating the oxidative stress caused by peroxides and other reactive oxygen species (Hillas *et al.,* 2000). Seemingly, the down-regulation of these proteins seemingly not be necessary to combat chlorhexidine stress may reflect the balanced utilisation of useful proteins to overcome chlorhexidine stress.

The DIGE data also suggests the possibility of detoxification or degradation of chlorhexidine by amidohydrolases. The amidohydrolase superfamily is functionally-diverse, metal-dependent proteins that contain a triosephosphate isomerase (TIM)-like barrel fold in their catalytic domains (Aimin *et al.,* 2007). They are important for amino acid and nucleotide metabolism, as well as biodegradation of industrial compounds, chemicals and pesticides. Although chlorhexidine treatment resulted in an increase in amidohydrolase abundance by 2-fold in both WT15 and MT51 biofilms (including proteins involved in protein synthesis, folding and stabilisation, such as GroEL, ClpP protease, aspartyl/glutamyl-tRNA amidotransferase, elongation factors Tu and Ts), chlorhexidine degradation studies using ^{14}C-radiolabelled chlorhexidine demonstrated that WT15 could not mineralize chlorhexidine (Rema *et al.,* 2014), though chlorhexidine degradation by other bacterial species is known (Tanaka *et al.,* 2005).

A few proteins associated with energy production, nucleotide transport and metabolism were also found to be up-regulated (Table 4.3) in the presence of chlorhexidine. Their increase in abundance may be due to the higher

energy demand for energy-dependent mechanisms of detoxification or adaptation to chlorhexidine. These proteins were also found to be more abundant in *B. cenocepacia* that was highly resistant to different classes of antimicrobials (Madeira *et al.,* 2011).

Consistent with the observed shift in fatty acid composition, several enzymes associated with fatty acid synthesis and metabolic processes (Table 4.3) were affected by chlorhexidine treatment in both strains. Microorganisms often adapt to environmental stress by changing the type and composition of membrane fatty acids (Sinensky, 1974) and the induction of these enzymes in *D. acidovorans* biofilms suggests this as a contributing adaptation mechanism to chlorhexidine. For example, increased expression of malonyl-CoA: ACP transacylase indicates increased fatty acid synthesis, which means increased consumption of acetyl-CoA as malonyl-CoA is derived from acetyl-CoA in the first step of fatty acid synthesis (Tong, 2005; Pyla *et al.,* 2010). Consumption of acetyl-CoA for such purposes would decrease the production of poly-β-hydroxybutyrate (PHB) in PHB-producing organisms such as *D. acidovorans* (Sudesh *et al.,* 1999), consistent with the observed decreased expression of phasin proteins which are known to be produced only during production of PHB (York *et al.,* 2002).

There is also a convincing indication of the involvement of several membrane proteins in chlorhexidine tolerance in *D. acidovorans* biofilms. A basic membrane lipoprotein (spot no. 371, Table 4.3), which is an outer membrane protein, was highly up-regulated in both WT15 and MT51 chlorhexidine-treated biofilms. Bacterial lipoproteins are known to have many functions, some of which include transport, signaling, antibiotic resistance, conjugation, protein secretion. Their involvement in microbial antibiotic resistance has been reported previously (Burns *et al.,* 1996; Chang *et al.,* 2012). A possible role for quorum sensing in the response to chlorhexidine is also suggested due to the down-regulation of the two-component LuxR family transcriptional regulator, especially in WT15 biofilms treated with chlorhexidine.

The DIGE analysis indicates that overall chlorhexidine tolerance in *D. acidovorans* is an outcome of the effects of various antimicrobial resistance mechanisms at the molecular level, as has previously been reported for triclosan resistance in *Salmonella enterica* serovar Typhimurium (Weber *et al.,* 2008). Of these, stress proteins,

chaperones and proteins involved in fatty acid metabolic processes, and possibly in membrane stability, appear to play a very important role in chlorhexidine tolerance in *D. acidovorans* biofilms.

5. GENERAL DISCUSSIONS AND CONCLUSION

Microorganisms can become 1000 times more resistant to antimicrobials when they grow as biofilms (Tremblay *et al.,* 2014). This becomes even more concerning when environmental microorganisms such as *D. acidovorans* that have been reported to be opportunistic pathogens are linked to antimicrobial resistance phenomena with limited data available on the actual mechanisms providing this enhanced tolerance. *Delftia acidovorans* is a Gram negative bacteria ubiquitously found in soil and water and has been associated with serious human infections (Cho and Lee, 2002; Perla and Kuntson, 2005) and infections associated with hospital devices (Weinstein *et al.,* 1976; Mino *et al.,* 2000; Kawamura *et al.,* 2011). Several studies using *D. acidovorans* have reported their antimicrobial resistance capabilities against commonly-used antibiotics (Kawamura *et al.,* 2011; Kam *et al.,* 2012; Khan *et al.,* 2012). Among biocides, chlorhexidine (CHX) is considered the gold standard against which other antibacterial and antiplaque agents are assessed (Mathur *et al.,* 2011). Several microorgansims have been found to be resistant to CHX (Stickler and Thomas (1980); Stickler, 2002); however, the exact mechanisms of CHX resistance are still not completely understood.

Various studies have shown that physiological mechanisms tend to operate in concert at different hierarchical levels, many times synergistically, in order to confer enhanced tolerance in biofilms exposed to any environmental stress factor (Mah and O'Toole, 2001; Szomolay *et al.,* 2005; Mangalappalli-Illathu and Korber, 2006). The adaptive or tolerance mechanisms to these stresses reportedly vary from cell-surface changes, cellular morphological modification, production of degradative enzymes or efflux proteins, alterations in cellular fatty acid composition, induction of other stress response proteins and formation of persister cells (Braga and Ricci, 1998; Szomolay *et al.,* 2005; Mangalappalli-Illathu and Korber, 2006). The research described in my thesis examined the effect of CHX on *D. acidovorans* biofilms at different concentrations, and is an attempt to gain knowledge about the underlying mechanisms that are involved in antimicrobial tolerance in this organism. I also further attempted to examine the role of a membrane protein, TolQ, in CHX tolerance in *D. acidovorans* biofilms. A deeper

mechanistic understanding will help in the development of compounds or other strategies that can interfere with antimicrobial tolerance development, and thus make *Delftia* biofilms susceptible to conventional antimicrobial compounds, enable the removal of biofilms in environmental settings and successful treatment of a variety of infections caused by these biofilm-forming bacteria. Broadly, some of the antimicrobial mechanisms shown to operate in microbial biofilms include: a) explusion of the agent by efflux systems, b) degradation, c) persister cell formation, d) penetration failure of the agent either due to altered cell membrane or biofilm properties etc.

In this research work, I used a strain of *D. acidovorans* that was isolated from a river source and had a CHX MIC of 15 μg ml^{-1} and was considered to be CHX tolerant (WT15). To my knowledge, this is the first report of *D. acidovorans* tolerance to a broad spectrum biocide such as CHX (Rema *et al.,* 2014) and adds to our knowledge regarding antimicrobial resistance a critical issue with regard to emerging environmental pathogens. This study further demonstrates that antimicrobial resistance among microbial species is environmentally relevant and is not just an issue in hospital settings. This study also provides an appropriate example for the development of tolerance in biofilms in not just commonly-encountered pathogens such as *E. coli*, *Salmonella*, *Listeria*, etc. and that tolerance may be found in emerging pathogens and other bacterial species that have received less attention by microbiologists. Strain WT15 readily formed biofilms with very minimal nutrient media (1% TSB) and could tolerate above MIC levels of CHX (30 μg ml^{-1}), as demonstrated in the studies published in Rema *et al.* (2014). This is in part due to the biofilm mode of growth providing increased tolerance to antimicrobials. Undoubtedly, this phenomenon is a serious concern with significant practical implications for the control of biofilms in healthcare facilities, households, food and other industries as routine cleaning procedures fail to eradicate them completely.

A CHX-susceptible strain MT51 (MIC-1 μg ml^{-1}) created by insertional mutation at the 81st bp of the *tolQ* gene indicated the putative role of this membrane protein in CHX tolerance in *D. acidovorans*. CHX is a membrane-active agent and mutation of a membrane protein (TolQ) that increased the susceptibility of *D. acidovorans* to CHX clearly points to a role of the cell membrane in CHX tolerance. Mutations in *tol* genes have previously been shown to confer hypersensitivity to various other agents, including detergents, quaternary

compounds, and some antibiotics in *E. coli*. This was further supported by the CLSM data that used fluorescent live-dead staining to demonstrate the effect of CHX based on the membrane permeability of CHX-susceptible and CHX-tolerant strains (Rema *et al.,* 2014). The uptake levels of CHX also varied between these strains as CHX was shown to accumulate to a greater extent inside CHX-susceptible mutant cells than in CHX-tolerant cells, as shown by STXM and IR analysis (Rema *et al.,* 2014). The cell membrane is the first barrier to entry of antimicrobial agents and any entry into the cells indicates the cell membrane has somehow been breached. The observation of two different cell types within WT15 biofilms, one that apparently does not, or only slowly, takes-up CHX relative to the other (Rema *et al.,* 2014) may indicate the existence of a "persister cells" within these biofilms and needs further validation. Ethylenediaminetetraacetic acid (EDTA) the outer membrane permeability of Gram negative bacteria by sequestering di- and multi-valent cations, and was shown to promote bioaccumulation of CHX in Delftia biofilms. Disruptions in cell membrane of cells exposed to CHX were also observed by TEM. Thus, all these studies point to the cell membrane as having a key role in CHX tolerance.

Further, to my knowledge this study is also the first to investigate global changes in protein expression patterns of *D. acidovorans* under the influence of CHX. As reported in several other proteomic stress-response studies, my research also showed that bacterial environmental stress response is a coordinated, pan-metabolic outcome of the expression of a variety of genes that alter various cellular processes (cell division, DNA metabolism, housekeeping, membrane composition, transport, etc.). Numerous protein species-abundance changes in both CHX-tolerant WT15 and CHX-susceptible MT51 biofilms were seen when exposed to sub-MIC (10 μg ml^{-1}) and above-MIC (30 μg ml^{-1}) levels of CHX. These changes were predominantly greater in CHX-treated mutant biofilms than the wild type biofilms, indicating some of involvment of *tolQ* in CHX tolerance. Gene ontology analysis revealed the collective participation of a wide range of key enzymes and proteins in CHX tolerance. These molecules were mainly involved in energy, nucleotide, fatty acid and amino acid metabolism, protein translation and modification, DNA binding and transcription, cell membrane-related functions and other cellular processes such as detoxification and stress response.

Overall, a multi-faceted, pan-metabolic response to CHX appears to exist in *D. acidovorans* biofilm cells (cellular detoxification, fatty acid and proteome modifications, synthesis of various bacterial stress response proteins, etc.), but primarily appears to be the cell envelope that ultimately influences the inhibition of *D. acidovorans* species by CHX. These stress responses may help the *D. acidovorans* biofilms to enhance their ability for survival in the nature and have a great significance especially if formed in critical environments such as healthcare facilities, the food industry, and households. There is further need to improve our understanding of the key factors influencing these enhanced tolerance mechanisms in order to develop effective control strategies in the future.

6. POSSIBLE FUTURE STUDIES

My thesis research offers intial insights into biofilm development by *Delftia acidovorans* and the role of *tolQ* and the cell membrane in CHX tolerance. The stress response of *D. acidovorans* biofilms challenged with CHX was also examined at the molecular level and revealed that several proteins involved in various biological processes contributed to overall CHX tolerance. However, due to certain limitations and scope of the work, there are still some areas that could be further investigated for a better understanding of CHX tolerance in microorganisms. These include:

1. Most of the data on CHX-resistant microorganisms is associated with clinical specimens; however, the problem is seemingly more widespread than currently believed. More data on the prevalence of CHX-resistant microorganisms in environments, other than those from clinical settings, are needed to more fully appreciate this reality as this, in turn, may impact the health of living beings and may be a contributing factor for further cross resistance as reported in case of some commonly used antibiotics and other biocides.

2. Apart from those used in this study, there may be other growth conditions that may enhance biofilm development in *D. acidovorans* and further increase CHX tolerance. Some examples include the availability of nutrients in media, media flow rate, type of substratum, age or thickness of biofilm before CHX exposure, etc.

3. A more detailed molecular characterization of the role of *tolQ* gene in CHX resistance in the *D. acidovorans* strains used in my study is needed to provide further insights into the CHX tolerance mechanism. The interaction of TolQ with other tol proteins also needs to be elucidated. This may also help to determine the effect of *tolQ* on other proteins that were differentially-expressed between the wild type and mutant strains when exposed to CHX.

4. It would be very interesting to see whether the two cell populations identified using STXM in CHX tolerant biofilms can be isolated and studied to confirm if CHX tolerance was a result of persister cell formation. A

correlative microscopic approach applying CLSM and STXM could be undertaken to see whether these two cell populations are the result of the status of membrane integrity in these cells.

5. Both FAME and DIGE analysis indicated that fatty acid metabolism or pathways were altered in CHX-exposed Delftia biofilms. Further investigation in this area, particularly examining the role of cyclic fatty acids in CHX tolerance, may be worthwhile as these fatty acids have been found to play an important role in bacterial stress response.

6. Due to the high cost involved in protein identification, I was limited to the identification of only 40 proteins that were differentially-expressed and thus my study may have missed reporting other proteins, or groups of proteins, that may be biologically-relevant to CHX tolerance. Many of the differentially expressed proteins can be further studied individually to understand their actual role in CHX tolerance in *Delftia* biofilms such as Clp protease, basic membrane lipoprotein, TauD and LuxR and based on that the actual mechanism of CHX tolerance can be narrowed down for example, efflux/ detoxification or fatty acid changes. Advanced gel free proteomic mass spectrometric techniques could be employed to comprehensively identify either the whole cell proteome or more specifically the membrane proteins to save time and to eliminate enrichment procedures.

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